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(54) Title: COMPOSITIONS FOR THE TREATMENT OF TUMORS, AND USES THEREOF

(57) Abstract

In accordance with the present invention, it has been discovered that the nuclear export signal of tumor suppressor proteins can be impeded from mediating export of the tumor suppressor protein from the cell nucleus. Accordingly, there are provided peptides which elevate tumor suppressor function within the cell nucleus, yet are impeded from exiting the cell nucleus to the cytoplasm. Invention peptides are impeded from being exported from the cell nucleus by alteration of a nuclear export signal of a tumor suppressor protein or by compounds which inhibit the nuclear export signal from complexing with the nuclear export machinery. In accordance with another embodiment of the present invention, there are provided methods for treating a neoplastic condition, such as tumors, by administering compounds which elevate tumor suppressor function within the cell nucleus.

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Compositions for the Treatment of Tumors, and Uses Thereof

FIELD OF THE INVENTION

The present invention relates to compositions which provide tumor suppressor function in the nucleus, as well as methods for the uses of such compositions for therapeutic purposes.

BACKGROUND OF THE INVENTION

Tumor suppressor genes (TSGs) are a class of genes involved in different aspects of normal control of cellular growth and division. A common characteristic of such genes is that inactivation thereof, usually by genetic means, contributes to tumor development. Tumor suppressor proteins are transcriptional regulators that are activated to induce cell cycle arrest or apoptosis when cells are challenged by a variety of genotoxic stresses. This contrasts with the other main class of genes involved in neoplasia - the dominantly acting oncogenes, whose activation, again by various genetic means, also leads to a malignant phenotype. To emphasize this contrast, and to reflect the possibility that TSGs directly antagonize the action of oncogenes, TSGs are sometimes referred to as anti-oncogenes. TSGs must usually be inactivated at both alleles for manifestation of their oncogenic effect, although there are examples of phenotypic effects at the cellular level in the heterozygous condition.

The first evidence for the existence of TSGs came from the unexpected observation that the fusion of normal cells with tumor cells resulted in hybrids which were nontumorigenic. However, with the loss of chromosomes, which occurs naturally when hybrid cells are grown in culture, the tumorigenic phenotype was sometimes restored. Furthermore, the introduction of normal chromosomes into tumorigenic hybrids can cause reversal to the nontumorigenic phenotype. Thus expression of TSGs from the normal parent is capable of suppressing tumorigenicity.

Since the normal functioning of TSGs suppresses tumorigenicity, regions of chromosomes which are lost in tumors are likely to carry these TSGs. Karyotype analysis of a wide variety of tumor types has shown that chromosomal deletions occur with a high frequency and in a tumor-specific manner, and thus point to the likely location of TSGs. RFLP analysis, which allows comparison of a patient's normal and tumor genotype, confirms the prevalence of allelic loss inferred from cytogenetic analysis. This approach allows sufficient localization of TSGs to highlight regions within chromosomes warranting further intensive study, and this has led to the isolation of several TSGs.

The study of allelotypes, besides identifying regions of allelic loss, has also been very important in revealing the underlying mechanism of tumor suppressor gene activation. Heritable retinoblastoma, for example, is a dominant condition which predisposes to retinal tumors at an early age. It turns out that it is the normal allele, from the unaffected parent, which is lost in the tumor. This loss of heterozygosity reveals an underlying mutation in the remaining chromosome. In the case of inherited tumors such as retinoblastoma, the mutant allele is inherited and the normal allele is lost by some somatic mechanism. This second event occurs with high frequency, explaining why the disease is inherited as a dominant trait, although at the cellular level the locus involved is recessive. For the 'sporadic' cases of these tumors which arise purely somatically, inactivation of TSGs requires genetic damage to both alleles at a tumor suppressor gene locus. This is frequently the result of mutation, or submicroscopic deletion, on one chromosome, and deletion or whole chromosome loss of the other homologous chromosome.

Several genes have been isolated which to a greater or lesser extent fulfill the criteria for a tumor suppressor gene. These are: (1) localization to a region of a chromosome showing consistent allelic loss; (2) genetic damage of the remaining allele (although inactivation may be by epigenetic means); and (3) growth inhibition or reduction in tumorigenicity on reintroduction of the gene into the tumor type in which it is inactivated. The gene isolated from a region deleted in retinoblastoma cells -- the

RB1 gene - most adequately meets these criteria, but other candidate TSGs which fulfill these criteria include *WT1* (a gene isolated from a region (11p13) occasionally deleted in inherited and somatically arising Wilms' tumor), *NF1* (a gene thought to be involved in the development of neurofibromatosis), *P53* (a gene which seems to be inactivated in a wide variety of tumors), *DCC*, *MCC* and *APC* (FAP) (all found to be involved in the development of colon cancer) and numerous others.

Although much attention has been focused on the effects of various post-translational modifications, evidence is accumulating that differential subcellular localization is an additional important mechanism for regulating tumor suppressor activity. The subcellular localization of tumor suppressor protein is established by energy-dependent shuttling between the nuclear and cytoplasmic compartments. For example, p53 has three well-characterized nuclear localization signals (NLS) which mediate entry into the nucleus through the nuclear pore (Shaulsky et al. (1990b) *Oncogene* **5(11)**:1707-1711). Mutation of the primary nuclear localization signal of p53 results in constitutive cytoplasmic localization and the resultant inability to suppress formation of rat embryonic fibroblast foci induced by E1A or ras (Shaulsky et al. (1991) *Cell Growth Differ* **2(12)**:661-667). The issue of how tumor suppressor proteins exit the nucleus has not previously been addressed.

Nuclear export of proteins is mediated by a nuclear export signal (NES), a leucine-rich peptide motif necessary for the formation of a complex with components of the nuclear export machinery, i.e., the export receptor, CRM1, and RanGTP which is capable of energy-dependent translocation through the nuclear pore (Wen et al., 1995; Fischer et al. (1995) *Cell* **82(3)**:475-483; Stade et al. (1997) *Cell* **90(6)**:1041-1050; Fornerod et al. (1997) *Cell* **90(6)**:1051-1060; Ossareh-Nazari et al. (1997) *Science* **278(5335)**:141-144. Nuclear export signals have been identified in an increasing number of proteins, including HIV Rev, PKI, IkBα, and MAPKK, and more recently MDM2 (Bogerd et al. *Mol Cell Biol* **16**:4207-4214; Fischer et al. (1995) cited above; Wen et al. (1995) cited above; Fukuda et al. (1996) *J Biol Chem* **271(33)**:20024-20028; Bachelerie et al. (1997) *J Cell Sci* **110(22)**:2883-2893; Roth et al. (1998) *EMBO J* **17(2)**:554-564.

Subsequent studies have demonstrated the importance of nuclear export as a crucial step in a variety of cellular processes, including generalized translocation of both cellular and viral RNAs and proteins, inhibition of signal-transducing enzymes through nuclear exclusion, and communication between the nucleus and the cytoplasm or extracellular matrix (Pasquinelli et al. (1997) *Proc Natl Acad Sci USA* **94(26)**:14394-14399; Arenzana-Seidedos et al. (1997); Fukuda et al., 1997; Nix and Beckerle, 1997; for review see Ullman et al., 1997).

Thus, there is a need in the art for the identification of compositions which provide tumor suppressor function in the nucleus, as well as for methods for the use of such compositions for therapeutic purposes.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been discovered that the nuclear export signal of tumor suppressor proteins can be impeded from mediating export of the tumor suppressor protein from the cell nucleus. Accordingly, there are provided peptides which elevate tumor suppressor function within the cell nucleus, yet are impeded from exiting the cell nucleus to the cytoplasm. Invention peptides are impeded from being exported from the cell nucleus by alteration of a nuclear export signal of a tumor suppressor protein or by compounds which inhibit the nuclear export signal from complexing with the nuclear export machinery. Maintainance of tumor suppressor function within the nucleus suppresses, inhibits and/or regulates the growth and differentiation of neoplastic cells.

In accordance with another embodiment of the present invention, there are provided methods for treating a neoplastic condition, such as tumors, by administering compounds which elevate tumor suppressor function within the cell nucleus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides a comparision of NES sequences. p53 contains a highly-conserved, rev-like NES. The p53 NES is aligned with a depiction of a consensus rev-like NES, with other published NESs, and with putative NESs from the recently described homologous p53 family members, p73 and KET.

Figure 2 is a diagram depicting the different domains within p53, including the general location of p53 NES within the C-terminus. The p53 NES lies within the A-helix portion of the tetramerization domain, which is required for the creation of tetramers from a pair of dimers. The NES also lies between two of the three characterized nuclear localization signals.

Figure 3 aligns the sequence of the wild type NES, as well as the 348 and 348/350 p53 NES mutants used in these studies.

Figure 4 is a map of the p53-GFP plasmid used in these studies. This map depicts the wt p53-GFP. The other mutants used in these studies, 22/23, 348, and 348/350-GFP were constructed in the same fashion.

Figure 5 is a graph depicting Saos-2 transfection, wt vs. NES-GFP constructs. Saos-2 osteosarcoma cells were co-transfected using Qiagen SuperFect transfection reagent with 20, 100, or 500 ng of wt p53-GFP, 348-GFP, 348/350-GFP, 22/23-GFP, or empty vector, and 750 ng of a WWP-luciferase reporter construct consisting of a p21 promoter driving the expression of firefly luciferase. The 22/23-GFP construct is incapable of transactivation and serves as a negative control. These data are representative of relative activity obtained in multiple experiments.

Figure 6A is a chart depicting the growth suppression by NES mutants in HS683 $p53^{-/-}$ glioblastoma cells. HS683 $p53^{-/-}$ glioblastoma cells were infected with p53-GFP, 348/350-GFP, 348-GFP, or 22/23-GFP containing retroviruses (at a multiplicity of

infection of 2 or 10) and incubated under G418 selection for 3 weeks, fixed in 3.7% formaldehyde, stained, and counted.

Figure 6B is a chart depicting the growth suppression by NES mutants in U251 p53 DNA-binding mutant glioblastoma cells. Colony suppression by wild type p53, as well as 348/350 and 348 NES mutants is expressed relative to 22/23, a transactivation mutant incapable of suppressing colony formation.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided peptides having tumor suppressor activity, wherein the peptide comprises a tumor suppressor protein having partially or fully impeded export function. In a preferred aspect of the present invention, the peptides are unable to associate with a component of the nuclear export machinery, such as CRM1 or RanGTP. In yet another aspect of the present invention, the peptides further comprise a nuclear export signal of a tumor suppressor protein inhibited from associating with components of the nuclear export machinery, thus inhibiting the peptide from exiting the cell nucleas. Preferably, such impedence or inhibition of export is caused by alteration of the nuclear export signal of tumor suppressor protein.

Tumor suppressor genes are genes whose absence, mutation or inactivation allows the malignancy of neoplasms to manifest. The absence, mutation or inactivation of tumor suppressor gene in a particular type of tumor can be detected in samples from almost all patients, and where it is not detected, this may be because of the detection strategy rather than the fact that these tumors are exceptional. This situation contrasts with that for the transforming oncogenes which are activated in only a small proportion of patients with a given tumor type. This is probably a reflection of the roles of these genes in tumor progression - tumor suppressor genes being more often involved in the early stages of tumor development.

As employed herein, the term "tumor suppressor protein" or "gene product" refers to the expressed polypeptide product of tumor suppressor genes. Tumor suppressor genes contemplated for use herein include, but are not limited to genes encoding p53 protein, p73 protein (Kaghad et al. (1997) *Cell* **90(4)**:809-819), KET (Schmale and Bamberger (1997) *Oncogene* **15**:1363-1367), retinoblastoma protein (Rb) (Goodrich et al. (1993) *Biochim Biophys Acta* **1155**:43-61, Hamel et al. (1993) *FASEB J* 7:846-854, Hollingsworth et al. (1993) *Curr Opin Genet Dev* **3**:55-62, Toguchida et al (1993) *Genomics* **17**:535-543), Wilm's Tumor (WT1) protein (Campbell et al (1994) *Oncogene* **9**:583-595) "deleted in colorectal carcinoma" protein (DCC), adenomatous polyposis coli protein (APC), "mutated in colorectal carcinoma" protein (MCC), Wilm's tumor 1 protein (WT1), neurofibromatosis type 1 or type 2 protein ((NF1 or NF2) (Marx (1993) *Science* **261**:1385–1387), von Hippel-Lindau Disease (VHL) protein (Latif et al. (1993) *Science* **260**:1317–1320, Wei et al. (1993) *Science* **260**:1317-1320), Multiple endocrine neoplasia type 1 and 2A (MEN1 and MEN2A), Neuroblastoma (NB1), MLM, Basal Cell Nevus Syndrome (BCNS), Renal Cell Carcinoma (RCC), Familial Breast Cancer type 1 and 2, (BRCA1 and BRCA2), ETS1, and the like (for general overview, see also McCormick et al. (1994) *Chem Biol* 1(1):7-9, Brown, MA (1997) *Adv Genet* 36:45-135, Berbec et al. (1997) *Postepy Hig Med Dosw* 51(3):269-284). Tumor suppressor protein (also referred herein as "tumor suppressor gene product") includes proteins from any species, including amphibian, reptilian, mammalian, (i.e., bovine, ovine, porcine, equine, and preferably human), in native-sequence or in variant form, and from any source, whether natural, synthetic, or recombinant. Also included within the scope of the present invention are analogs, homologs and mimics of tumor suppressor genes.

As used herein, the term "nuclear export function" refers to the ability of the nuclear export signal to mediate export of polypeptides from the cell nucleus into the cytoplasm. NES mediate nuclear export function by complexing with components of the nuclear export machinery (Fornerod et al. (1997) Cell **90**:1051-1060). As used herein, the term "components of the nuclear export machinery" refers to those polypeptides which associate with NES-bound protein and facilitate export of such

protein from the cell nucleus into the cytoplasm. Examples of components include CRM1, RanGTP, and the like.

The amino acid sequences of nuclear export signals in tumor suppressor genes are conserved across species (i.e., p53 - Figure 1), implying that export function is also conserved. For example, analysis of Xenopus and human p53 proteins has revealed no differences. Thus, as seen in Figure 1 and Example 10, the nuclear export signal is also highly conserved among various species. Similarly, as seen in Figure 1, the NESs (putative) are highly conserved among different tumor suppressor genes. Those of skill in the art can readily identify additional NESs based on the disclosure of Examples 10 and 11. For example, for nuclear export signals for tumor suppressor proteins that are not listed in either Figure 1 or Example 10, those of skill in the art can readily identify additional NESs by alignment of putative NESs to the disclosed NESs and those known in the art by computer programs commercially available and known in the art, i.e., such as the program BESTFIT (University of Wisconsin GCG package Rechid et al. (1989) *Comput Appl Biosci* **5(2)**:107-113). Determination of nuclear export function can be determined by methods known in the art or by the method disclosed in Example 11.

As employed herein, the term "altered" or derivatives thereof, as employed to characterize tumor suppressor NES, refers to mutations, substitutions, deletions, additions and/or modifications to amino acid residues thereof. In a preferred aspect of the present invention, there are provided alterations to the NES, most preferably to the hydrophobic residues within the NES. The experiments disclosed herein reveal that it is possible to mutate one or more of the hydrophobic amino acids and still retain tumor suppressor activity. For example, with respect to human p53, preferred alterations to the NES include alterations of amino acid residue 344, 348 and/or 350. Such alterations to human p53 include substituting one or more of the leucines within the NES with alanine. Other hydrophobic residues (within other tumor suppressors) which can be altered include those in Figure 1 and Example 10.

In a preferred embodiment of the invention, invention peptides are the result of replacing the native hydrophobic residue(s) of tumor suppressor genes with non-hydrophobic residues. Alternatively, the hydrophobic residues of invention peptide(s) can be further modified to affect hydrophobicity, i.e., by methylation, phosphorylation, and the like. By altering the NES of native tumor suppressor protein, the ability of the resulting peptide to export outside the nucleus (i.e., nuclear export function) is diminished or eliminated. Therefor, the concentration of peptide localized within the nucleus is increased, thereby increasing tumor suppressor function. In yet another aspect of the present invention, amino acid residues outside the NES can also be altered in order to diminish or eliminate tumor suppressor function. For example, with respect with p53, increase of the tetramerization activity of p53 can diminish or eliminate nuclear export function.

Peptides according to the invention can also be subjected to a variety of modifications. These modifications can be trivial (defined as having no effect on function) or beneficial (i.e. they improve upon some functional property of the protein), and can include deletions, insertions, amino acid substitutions and/or replacement of functional domains or regions of functional domains by functionally equivalent domains or regions of other proteins. It is understood that the proteins of the invention can contain more than one such modification, as described in greater detail herein.

Other modifications of the tumor suppressor proteins described herein include amino acid substitutions, deletions and insertions. Such modifications can enhance function or introduce a useful property. For example modifications contemplated by the present invention include introducing a tag to optimize protein purification (Scopes (1994), Protein Purification, Principles and Practice, third edition, Springer-Verlag, New York), or enhancing expression and/or stability of an invention peptide when expressed in vitro or in a patient. Modifications in invention peptide(s) can enhance DNA binding and growth suppressing activities. Two such modifications contemplated include, with respect to p53: substitution of arginine 174 with glutamine or of arginine 175 with leucine (the numbering refers to human p53; in mouse p53 the corresponding residues

are 171 and 172, respectively (Halazonetis and Kandil (1993) *EMBO J* **12(13)**:5057-5064.; Li et al. (1994) *Cell Growth Differentiation* **5**:711–721).

In a particular aspect of the present invention, invention peptide(s) further comprise heterologous oligomerization domain(s) (Waterman et al. (1995) *EMBO J* **14(3)**:512-519). Such heterologous oligomerization domains either replace or supplement the native oligomerization domain. Preferably, the replacement oligomerization domain lacks full or partial export function yet retains partial or full oligomerization function. Heterologous dimerization domains can readily be selected from a variety of proteins, such as the leucine zipper (LZ) element, i.e, GCN4, the retinoic acid receptor, the thyroid hormone receptor or other nuclear hormone receptors (Kurokawa et al. (1993) *Genes Dev* 7:1423–1435) or from the yeast transcription factors Gal**4** and HAP**1** (Marmonstein et al. (1992) *Nature* **356**:408–414**;** Zhang et al. (**1993**) *Proc Natl Acad Sci USA* **90**:2851–2855). Those of skill in the art can readily identify additional suitable dimerization domains, including artificial dimerization domains (O'Shea et al. (1992) *Cell* **68**:699–708; Krylov et al. (1994) *EMBO J* **13**:2849–2861).

In another particular aspect of the present invention, there are provided invention peptide(s) containing substitutions of functional domains or regions thereof with equivalent domains or regions of other proteins. Substitution of a functional domain can involve all or part of the domain. As an example of this type of modification, the p53 transactivation domain (amino acids 1-90 of human p53) can be substituted with the activation domain of another protein, e.g., VP16, GAL4, and the like. The advantage of such substitutions are that in certain tumors, negative suppressors (such as Mdm2 protein) suppress tumor suppressor protein-mediated transcription by masking its transactivation domain (Oliner et al. (1992) *Nature* **358(6381)**:80-83; Momand et al. (1992) *Cell* **69(7)**:1237-1245; Oliner et al. (1993) *Nature* **362(6423)**:857-860). For example, wild type mdm2 protein will bind and inhibit the activity of wild type p53. However, when the transactivation domain of p53 is replaced by that of VP16, it is no longer inhibited by Mdm2, because Mdm2 does not suppress the transcriptional activity mediated by VP16 (Oliner et al. (1993), supra).

Modifications in p53 can also affect interaction with cellular or viral proteins. For example, substitution of leucine 14 and phenylalanine 19, of amino acid sequence for human p53 protein, with glutamine and with serine, respectively, abolish the p53-Mdm2 interaction (Lin et al. (1994) *Genes Dev* **8(10)**:1235-1246).

Modifications in the heterologous oligomerization domain can increase the stability of oligomer formation. For example, substitutions that stabilize oligomerization driven by leucine zippers are known (Krylov et al. (1994) *EMBO J* **13(12)**:2849-2861; O'Shea et al. (1992) *Cell* **68(4)**:699-708.). As an exemplary modification of this type, residues 174 or 175 of human p53 are substituted by glutamine or leucine, respectively.

In a preferred aspect of the present invention, invention peptides further comprise nuclear localization signals (NLS), in addition to NLS native or endogenous to wild type tumor suppressor protein. For example, nuclear localization is required for p53 function (Shaulsky et al. (1991) *Oncogene*, **6**:2056–2065). Wild-type p53 contains three nuclear localization signals (NLS), all of which map to the C-terminus of wild-type p53 and specifically to residues 316-325, 369-375 and 379-384 of p53 (Shaulsky et al. (1990) *Mol Cell Biol* **10**:6565–6577). Some tumor suppressor proteins native to tumor cells lack one or more of these NLS. Thus, such proteins have an impaired ability to localize to the nucleus and consequently their function is impaired. It is therefore beneficial to reintroduce NLS into invention peptides. Even tumor suppressor proteins that maintain all native NLS can benefit from introduction of additional NLS. Therefore included within the scope of invention peptide(s) described herein are tumor suppressor proteins containing an NLS fused thereto. The added NLS can be introduced at the N-terminus, C-terminus, or at the junction of the transactivation and DNA binding domains or, with respect to the p53 protein, at the junction of the DNA binding and tetramerization domains or elsewhere in the protein, as long as the function of the tumor suppressor protein is not disrupted by insertion of the NLS. The NLS can be that of any tumor suppressor protein or of any other nuclear protein, such as the NLS of SV40 large T antigen (Kalderon et al. (1984) *Cell* **39**:499–509). Additional heterologous NLS are described by Shaulsky et al. ((1990), (1991) supra).

In accordance with another embodiment of the present invention, there are provided methods for treating a neoplastic condition in a subject in need thereof, said method comprising administering to said subject an effective amount of invention peptide, or nucleic acid encoding same. Invention peptides, or nucleic acid encoding same, can readily be employed to increase tumor suppressor function in cancer cells. Such use is appropriate in both cancerous or precancerous cells, in which the level of tumor suppressor protein is absent or diminished compared to normal cells. Invention peptides, or nucleic encoding same, can also be useful to increase the level of expression of tumor suppressor function even in those tumor cells in which tumor suppressor protein is expressed at a "normal level", but does not provide sufficient tumor suppressor function to allow normal cell growth/differentiation.

Furthermore, therapy involving the administration of invention peptide(s), or vector capable of expressing invention peptide(s), can be combined with other therapies for the disease or condition being treated, for example, with conventional cancer therapies, including surgery, radiation and chemotherapy.

In a preferred embodiment of the present invention, invention peptides, or nucleic encoding same, are employed to restore tumor suppressor function in tumor cells. Introduction of tumor suppressor function in tumor cells leads to decrease or arrest of cell proliferation (or the growth, dissemination or metastasis of tumors) or to cell death (see, e.g., p53 - Nielsen and Maneval (1998) *Cancer Gene Ther* **5(1)**:52-63; RB - Lee and Lee (1997) *Gan To Kagaku Ryoho* **24(11)**:1368-1380; BRCA1 - Shao et al. (1996) *Oncogene* **13(1)**:1-7). Invention peptides have general activity (inhibiting all new tumor formation) or alternatively have a specific activity (inhibiting the distribution, frequency, grade, etc.) of specific types of tumors in specific organs and tissue. Desirably, a suitable amount of the invention peptide is administered systemically, or locally to the site of the tumor with or without concurrent administration of conventional cancer therapy (i.e. DNA damaging agents, surgery, radiation, chemotherapy, and the like).

As used herein, the term "tumor suppressor function" (or "tumor suppressor activity") refers to the ability of invention peptide(s) to inhibit, suppress and/or regulate growth and differentiation of cells (i.e., relative to non-neoplastic cells of the same histological type). Therefor, "loss of tumor suppressor function" refers to the inability of tumor suppressor protein to regulate cell growth and differentiation, often leading to tumorigenicity. Tumor suppressor functions are lost by production of an inactive (i.e., mutant) form of tumor suppressor proteins or by a substantial decrease or total loss of expression of tumor suppressor proteins. Tumor suppressor functions are also substantially absent in neoplastic cells which comprise tumor suppressor alleles encoding wild-type tumor suppressor protein. For example, a genetic alteration outside of the tumor suppressor locus, such as a mutation that results in aberrant subcellular processing or localization of tumor suppressor protein (e.g., a mutation resulting in localization of tumor suppressor protein predominantly in the cytoplasm rather than the nucleus) can result in a loss of tumor suppressor function.

The particular function associated with each tumor suppressor gene is known to those of skill in the art. For example, as used herein, the term "Rb function" or "p53 function" refers to the property of having an essentially normal level of a polypeptide encoded by the RB or p53 gene (respectively) (i.e., relative to non-neoplastic cells of the same histological type), wherein the RB or p53 polypeptide (respectively) is capable of binding an E1a protein of wild-type adenovirus **2** or **5**. In general, tumor suppressor proteins act as cell cycle checkpoints, each regulating different points, for regulation of cells and differentiation of certain tissues. For example, RB and p53 act as cell cycle checkpoints at the G1 phase of various tissues. WT1, on the other hand, is tissue specific as it functions to regulate cell differentiation in the kidney. In addition, the characteristic neoplasm associated with each tumor suppressor gene is known to those of skill in the art. For example *RB1* inactivation is seen not just in retinoblastoma, but in many other tumor types including sarcomas, breast tumors, ovary tumors, bladder carcinoma, lung tumors, and the like. Similarly, *P53* is inactivated in probably an even wider range of tumors, including Sarcoma, glioma, breast carcinoma, and the like. Other exemplary tumors associated with specific tumor suppressor genes include: BRCA (1 and 2) with

breast and ovary carcinoma, NF (1 and 2) with tumors of the nervous system, WT1 with Wilm's tumor, and the like.

As used herein, "neoplastic cells" and "neoplasia" refer to malignant as well as non-malignant cell populations which often appear to differ from the surrounding tissue both morphologically and genotypically. Such cells exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells comprise cells which are actively replicating or in a temporary non-replicative resting state (G_1 or G_0); similarly, neoplastic cells can comprise cells which have a well-differentiated phenotype, a poorly-differentiated phenotype, or a mixture of both type of cells. Thus, not all neoplastic cells are necessarily replicating cells at a given timepoint. In addition, with respect to cells that are malignant, neoplastic cells exhibit the ability to metastasize (i.e., to invade other tissues). Malignant neoplastic cells are frequently referred to as cancer, typically termed carcinoma if originating from cells of endodermal or ectodermal histological origin, or sarcoma if originating from cell types derived from mesoderm. The development of a fully malignant cancer is thought to be a multistep process, involving at some state one or more mutations which alter genes (i.e., tumor suppressor genes) involved in the control of cell growth and differentiation.

A wide variety of cancer, papillomas and warts are contemplated for treatment by the same therapeutic strategy. Representative examples of cancers include colon, carcinoma, prostate cancer, breast cancer, lung cancer, skin cancer, liver cancer, bone cancer, ovary cancer, pancreas cancer, brain cancer, head and neck cancer, as well as other solid tumors, and the like. Representative examples of papillomas include squamous cell papilloma, choroid plexus papilloma, laryngeal papilloma, and the like. Representative examples of wart conditions include genital, plantar, epidermodysplasia verruciformis, malignant warts, and the like.

As employed herein, the term "subject" refers to the cell, tissue, organ or organism characterized by insufficient wild-type tumor suppressor gene activity. Preferably, subjects are mammals, or mammalian derived cells or tissue. Exemplary mammals include: humans; domesticated animals, e.g., rat, mouse, rabbit, canine, and feline; farm animals, e.g., chicken, bovine, porcine and ovine; and animals of zoological interest, e.g., monkey and baboon; and the like. The preferred mammal contemplated for treatment according to the invention is a human. Adults as well as non-adults (i.e., neo-nates, pre-pubescent mammals, and the like) are contemplated for treatment in accordance with the invention.

The compound (i.e., invention peptides or compounds which inhibit export thereof) can also be formulated into pharmaceutical compositions and administered using a therapeutic regimen compatible with the particular formulation. As a general proposition, the total pharmaceutically effective amount of compounds administered parenterally per dose will be an amount sufficient to provide a therapeutic effect (to arrest the growth of cancer cells) without inducing a significant level of toxicity. Since individual subjects present a wide variation in severity of symptoms and each form of invention peptide has its unique therapeutic characteristics, it is up to the practitioner to determine a subject's response to treatment and vary the dosages accordingly.

Administering to a subject contemplated by the present invention can be accomplished in a variety of ways, and the treating agents contemplated for use herein can be administered in a variety of forms (e.g., in combination with a pharmaceutically acceptable carrier therefor) and by a variety of modes of delivery. Exemplary pharmaceutically acceptable carriers include carriers suitable for oral, intravenous, subcutaneous, intramuscular, intraperitoneally, intravascularly, intracutaneous, and the like administration. Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, and the like, is contemplated.

For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing additives such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents, and the like.

For the preparation of fluids for parenteral administration, suitable carriers include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Alternatively, suspensions of the active compounds can be administered in suitable conventional lipophilic carriers or in liposomes.

The compositions can be supplemented by active pharmaceutical ingredients, where desired. Optional antibacterial, antiseptic, and antioxidant agents in the compositions can perform their ordinary functions. Such dosage forms can also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They can be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use.

Therapeutic compositions containing suitable compound are preferably administered intravenously, as by injection of a unit dose, for example. The term "unit dose," when used in reference to a therapeutic composition of the present invention, refers to a quantity of compound suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle. It can be particularly advantageous to administer such compounds in depot or long-lasting form as discussed hereinafter.

The required dosage will vary with the particular treatment desired, the degree and duration of therapeutic effect desired, the judgment of the practitioner, as well as properties peculiar to each individual. Moreover, suitable dosage ranges for systemic application depend on the route of administration. It is anticipated that a pharmaceutical composition containing active compound is generally effective for therapeutic treatment in dosages above about 0.1 mg per kg of body weight (mg/kg), preferably from about 1 milligram to about 100 milligram per kilogram of body weight per day being preferred. Dosage units of such pharmaceutical compositions containing the proteins of this invention preferably contain about 1 mg to 5 g of the protein. These doses can be administered with a frequency necessary to achieve and maintain satisfactory tumor suppressor activity levels.

Suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated. If given continuously, the composition is typically administered at a dose rate in the range of about 0.5 μg/kg/hour up to about 10 μg/kg/hour, either by 1-2 injections per day or by continuous subcutaneous release, for example, using a minipump, patch, implant, depot formulation, or the like.

In accordance with another aspect of the present invention, invention nucleic acids can also be introduced into subjects characterized by insufficient wild-type tumor suppressor gene activity, i.e. neoplastic diseases, to impart therapeutic benefits. The nucleic acid sequence encoding the invention peptide can be introduced into the patient in the form of a suitable vector or by direct DNA delivery to harness the patient's cellular machinery to express the proteins of the invention in vivo.

Nucleic acid can be stably incorporated into cells or can be transiently expressed using methods known in the art. For example, invention nucleic acid sequences can be inserted into a vector capable of targeting and infecting a desired cell, either *in vivo* or

ex vivo for gene therapy, and causing the encoded invention peptide to be expressed by that cell. Cells are cultivated under growth conditions (as opposed to protein expression conditions) until a desired density is achieved.

Gene transfer vectors can be introduced which express invention peptides in suitable host cells. In addition, stably transfected mammalian cells can be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene (such as the *E. coli* ß-galactosidase gene) to monitor transfection efficiency. Selectable marker genes are typically not included in the transient transfections because the transfectants are typically not grown under selective conditions, and are usually analyzed within a few days after transfection.

The amount of exogenous nucleic acid introduced into a host can be varied by those of skill in the art. For example, when a viral vector is employed to achieve gene transfer, the amount of nucleic acid introduced can be increased by increasing the amount of plaque forming units (PFU) of the viral vector. The number of copies of regulatory elements can readily be varied by those of skill in the art. For example, transcription regulatory regions can contain from 1 up to about 50 copies of a particular regulatory element, preferably 2 up to about 25 copies, more preferably 3 up to about 10-15 copies, with about 4-6 copies being especially preferred.

Gene transfer vectors (also referred to as "expression vectors") contemplated for use herein are recombinant nucleic acid molecules that are used to transport nucleic acid into host cells for expression and/or replication thereof. Such gene transfer vectors comprise nucleic acid sequences encoding invention peptide operatively associated with a desired promoter. Expression vectors can be either circular or linear, and are capable of incorporating a variety of nucleic acid constructs therein. Expression vectors typically come in the form of a plasmid that, upon introduction into an appropriate host cell, results in expression of the inserted nucleic acid.

As used herein, the phrase "operatively associated with" refers to the functional relationship of nucleic acid with regulatory sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of nucleic acid to a promoter refers to the physical and functional relationship between the nucleic acid and promoter such that the transcription of such nucleic acid is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the nucleic acid.

Promoters contemplated for control of expression of exogenous nucleic acids employed in the practice of the present invention include inducible, constitutive, and/or tissue specific promoters, preferably, inducible or tissue specific promoters. By employing an inducible promoter, one can control the expression of the gene encoding an invention peptide. The tumor suppressor gene can be expressed in patients upon exposure to endogenous inducers (such as trace metals or other factors found in serum for a metallothionein promoter or hormones for hormone-inducible promoters) or upon administration of exogenous inducers to the patients (e.g., allolactose for the lac operator-containing promoters). Inducible promoters contemplated for use in the practice of the present invention comprise transcription regulatory regions that function maximally to promote transcription of mRNA under inducing conditions. Examples of suitable inducible promoters include DNA sequences corresponding to: the *E. coli* lac operator responsive to IPTG (see Nakamura et al., *Cell*, 18:1109-1117, 1979); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g., zinc) induction (see Evans et al., U.S. Patent No. 4,870,009), the phage T7lac promoter responsive to IPTG (see Studier et al., *Meth. Enzymol.*, 185: 60-89, 1990; and U.S. #4,952,496), the heat-shock promoter; the TK minimal promoter; the CMV minimal promoter; a synthetic promoter such as a modified MMLV LTR; and the like.

Exemplary constitutive promoters contemplated for use in the practice of the present invention include the CMV promoter, the SV40 promoter, the DHFR promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, elongation factor 1α (EF1α) promoter, albumin

promoter, APO A1 promoter, cyclic AMP dependent kinase II (CaMKII) promoter, keratin promoter, CD3 promoter, immunoglobulin light or heavy chain promoters, neurofiliment promoter, neuron specific enolase promoter, L7 promoter, CD2 promoter, myosin light chain kinase promoter, HOX gene promoter, thymidine kinase (TK) promoter, RNA Pol II promoter, MYOD promoter, MYF5 promoter, phophoglycerokinase (PGK) promoter, Stf1 promoter, Low Density Lipoprotein (LDL) promoter, chicken b-actin promoter (used in conjunction with ecdysone response element) and the like.

As readily understood by those of skill in the art, the term "tissue specific" refers to the substantially exclusive initiation of transcription in the tissue from which a particular promoter, which drives expression of a given gene, is derived (e.g., expressed only in T-cells, endothelial cells, smooth muscle cells, and the like). Exemplary tissue specific promoters contemplated for use in the practice of the present invention are shown in table I.

Table I

TISSUE	PROMOTERS
liver	albumin, alpha-fetoprotein, α1-antitrypsin, transferrin transthyretin
colon	carbonic anhydrase I
ovary, placenta	estrogen, aromatase cytochrome P450, cholesterol side chain cleavage P450, 17 alpha-hydroxylase P450
prostate	prostate specific antigen, gp91-phox gene, prostate-specific kallikrein (hKLK2), prostate specific enhancer/promoter (PSE)
breast, G.I.	erb-B2, erb-B3, β-casein, β-lactoglobulin, WAB (whey acidic protein),
lung	surfactant protein C Uroglobin (cc-10, Cllacell 10 kd protein)
skin	K-14-keratin, human keratin 1 or 6, loicrin

brain	glial fibrillary acidic protein, mature astrocyte specific protein, myelin, tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), nerve growth factor (NT-3), BDNF
pancreas	villin, glucagon, insulin, islet amyloid polypeptide
thyroid	thyroglobulin, calcitonin
bone	Alpha 1 (I) collagen, osteocalcin, bone sialoglycoprotein
kidney	renin, liver/bone/kidney alkaline, phosphatase, erythropoietin (epo)

Additional components which can optionally be incorporated into expression vectors include selectable markers and genes. Selectable markers contemplated for use in the practice of the present invention include antibiotic resistance genes, genes which enable cells to process metabolic intermediaries, and the like. Exemplary antibiotic resistance genes include genes which impart tetracycline resistance, genes which impart ampicillin resistance, neomycin resistance, hygromycin resistance, puromycin resistance, and the like.

Genes which enable cells to process metabolic intermediaries include genes which permit cells to incorporate L-histidinol, genes encoding thymidine kinase, genes encoding xanthine-guanine phosphoribosyl transferase (gpt), genes encoding dihydrofolate reductase, genes encoding asparagine synthetase, and the like.

Suitable expression vectors for use herein include a recombinant DNA or RNA construct(s), such as plasmids, phage, recombinant virus or other vectors that, upon introduction into an appropriate host cell, result(s) in expression of the inserted DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

Expression vectors suitable for use in the practice of the present invention are well known to those of skill in the art and include those that are replicable in eukaryotic

cells and/or prokaryotic cells as well as those that remain episomal and those that integrate into the host cell genome. Expression vectors typically further contain other functionally important nucleic acid sequences encoding antibiotic resistance proteins, and the like.

Exemplary eukaryotic expression vectors include eukaryotic constructs, such as the pSV-2 gpt system (Mulligan et al., (1979) *Nature*, 277:108-114); pBlueScript (Stratagene, La Jolla, CA), the expression cloning vector described by Genetics Institute (*Science* (1985) **228**:810-815), and the like. Each of these plasmid vectors are capable of promoting expression of the receptor peptide of interest.

Suitable means for introducing (transducing) expression vectors containing invention nucleic acid constructs into host cells to produce transduced recombinant cells (i.e., cells containing recombinant heterologous nucleic acid) are well-known in the art (see, for review, Friedmann (1989) *Science* **244**:1275-1281; Mulligan (1993) *Science* **260**:926-932, each of which are incorporated herein by reference in their entirety). Exemplary methods of transduction include, e.g., infection employing viral vectors (see, e.g., U.S. Patent 4,405,712 and 4,650,764), calcium phosphate transfection (U.S. Patents 4,399,216 and 4,634,665), dextran sulfate transfection, electroporation, lipofection (see, e.g., U.S. Patents 4,394,448 and 4,619,794), cytofection, particle bead bombardment, and the like. The transduced nucleic acid can optionally include sequences which allow for its extrachromosomal (i.e., episomal) maintenance, or the transduced nucleic acid can be donor nucleic acid that integrates into the genome of the host.

In a specific embodiment, a gene transfer vector contemplated for use herein is a viral vector, such as Adenovirus, adeno-associated virus (AAV), or herpes-simplex virus based vectors, and synthetic vectors for gene therapy, and the like (see, e.g., Suhr et al. (1993) *Arch of Neurol* **50**:1252-1268, Schreiber et al. (1993) *Biotechniques* **14**:818–823, Davidson et al. (1993) *Nature Genetics* **3**:219–223, Roessler et al. (1993) *J. Clin. Invest* **92**:1085–1092, Smythe et al. (1994) *Ann Thorac Surg* **57**:1395–1401, Kaplitt et al. (**1994**) *Nature Genetics* **8**:148–154). There has already been success using

viral vectors driving expression of wild-type tumor suppressor genes (see, e.g., p53: Fujiwara et al. (1993) *Cancer Res* **53**:4129–4133, Fujiwara et al. (1994) *Cancer Res* **54**:2287–2291, Friedmann (1992) *Cancer* **70(6** Suppl):1810–1817, Fujiwara et al. (1994b) *Curr Opin Oncol* **6**:96–105).

Preferably, a gene transfer vector employed herein is a retroviral vector. Retroviral vectors are gene transfer plasmids that have an expression construct containing an exogenous nucleic acid residing between two retroviral LTRs. Retroviral vectors typically contain appropriate packaging signals that enable the retroviral vector, or RNA transcribed using the retroviral vector as a template, to be packaged into a viral virion in an appropriate packaging cell line (see, e.g., U.S. Patent 4,650,764).

Suitable retroviral vectors for use herein are described, for example, in U.S. Patents 5,399,346 and 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, each of which is hereby incorporated herein by reference, in their entirety. These documents provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e.g., Shackleford et al. (1988) *PNAS, USA*, **85**:9655-9659), human immunodeficiency virus (e.g., Naldini et al. (1996) *Science* **272**:165-320), and the like.

Various procedures are also well-known in the art for providing helper cells which produce retroviral vector particles which are essentially free of replicating virus. See, for example, U.S. Patent 4,650,764; Miller *Human Gene Therapy* **1**:5-14 (1990); Markowitz et al. *Journal of Virology* **61(4)**:1120-1124 (1988); Watanabe et al. *Molecular and Cellular Biology* **3(12)**:2241-2249 (1983); Danos et al. *PNAS* **85**:6460-6464 (1988); and Bosselman, et al. (1987) *Molecular and Cellular Biology* **7(5)**:1797-1806, which disclose procedures for producing viral vectors and helper cells which minimize the chances for producing a viral vector which includes a replicating virus.

Recombinant retroviruses suitable for carrying out the invention methods are produced employing well-known methods for producing retroviral virions. See, for example, U.S. Patent 4,650,764; Miller (1990) *Human Gene Therapy* **1**:5-14; Markowitz et al. (1988) *Journal of Virology*, **61(4)**:1120-1124; Watanabe et al. (1983) *Molecular and Cellular Biology* **3(12)**:2241-2249; Danos et al. *PNAS* **85**:6460-6464 (1988); and Bosselman, et al. (1987) *Molecular and Cellular Biology* **7(5)**:1797-1806.

Nucleic acid sequences driving expression of invention peptide can also be introduced by "carriers" other than viral vectors, such as liposomes, nucleic acid-coated gold beads or can simply be injected in situ (Fujiwara et al (1994b) supra; Fynan et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**:11478–11482, Cohen (1993) *Science* **259**:1691–1692; Wolff et al. (1991) *Biotechniques* **11**:474–485).

Typically, nucleic acid sequence information for tumor suppressor genes contemplated for use employed herein can be located in one of many public access databases, e.g., GENBANK, EMBL, Swiss-Prot, and PIR, or in related journal publications. Thus, those of skill in the art have access to sequence information for virtually all known tumor suppressor genes. Those of skill in the art can either obtain the corresponding nucleic acid molecule directly from a public depository or the institution that published the sequence. Optionally, once the nucleic acid sequence encoding a desired protein has been ascertained, the skilled artisan can employ routine methods, e.g., polymerase chain reaction (PCR) amplification, to isolate the desired nucleic acid molecule from the appropriate nucleic acid library. Thus, all known nucleic acids encoding proteins of interest are available for use in the methods and products described herein.

Genes encoding proteins required for retroviral packaging, e.g., the *pol* gene, the *gag* gene and the *env* gene are also contemplated for incorporation into expression vectors. For example, a recombinant viral vector, e.g. an adenovirus, of the present invention comprises DNA of at least that portion of the viral genome which is capable of infecting the target cells operatively linked to the nucleic acid sequences of the

invention. By "infection" is generally meant the process by which a virus transfers genetic material to its host or target cell. Preferably, the virus used in the construction of a vector of the invention is rendered replication-defective to remove the effects of viral replication on the target cells. In such cases, the replication-defective viral genome can be packaged by a helper virus in association with conventional techniques.

Although, as indicated above, such therapy can be provided to a recipient in order to treat (i.e. suppress, attenuate, or cause regression) an existing neoplastic state, the principles of the present invention can be used to provide a prophylactic gene therapy to individuals who, due to inherited genetic mutations, or somatic cell mutation, contain cells having impaired tumor suppressor gene expression (for example, only a single functional allele of the tumor suppressor gene) or impaired tumor suppressor function (for example, due to mutation of the tumor suppressor gene). Such therapy would be administered in advance of the detection of cancer in order to lessen the individual's predisposition to the disease.

In accordance with another embodiment of the present invention, there are provided methods for elevating the level of tumor suppressor gene product in the nucleus of a cell, said method comprising contacting said cell with a compound capable of inactivating export of said tumor suppressor gene product, thereby blocking transport of tumor suppressor gene product from the nucleus. Functional modulation of nuclear export of tumor suppressor protein includes partial to complete inhibition of the nuclear export signal of the tumor suppressor protein. Compounds which inhibit or block export of tumor suppressor protein include antibodies, peptides, oligonucleotides and small organic molecules, including peptidomimetics, which bind to the NES of tumor suppressor protein or inhibit the NES from binding components of nuclear export machinery, or which bind to components of the nuclear export machinery (i.e., CRM1).

In another aspect of the present invention, there are provided methods for suppression of cell proliferation, said method comprising contacting said cell with a compound capable of inactivating the nuclear export signal of a tumor suppressor gene

product, thereby blocking transport of tumor suppressor gene product from the nucleus. Compounds which inhibit or block export of the tumor suppressor gene product include antibodies, peptides, oligonucleotides and small organic molecules including peptidomimetics.

Antibodies useful for modulating nuclear export of tumor suppressor protein include both polyclonal and monoclonal, directed to epitopes corresponding to the nuclear export signal of tumor suppressor proteins. If polyclonal antibodies are desired, a selected mammal such as a mouse, rabbit, goat or horse is immunized with a protein of the present invention, or its fragment, or a mutant protein. Serum from the immunized animal is collected and treated according to known procedures. Serum polyclonal antibodies can be purified by immunoaffinity chromatography or other known procedures.

Monoclonal antibodies to the nuclear export signal of tumor suppressor proteins can also be readily produced by those skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al. *Hybridoma Techniques* (1980); Hammerling et al., *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al. *Monoclonal Antibodies* (1980); and U.S. Pat. Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the antigen of interest, or fragment thereof, can be screened for various properties, i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in inhibiting or interfering with the nuclear export of tumor suppressor gene products.

Another aspect of the invention is single-chain intracellular antibodies against the nuclear export signal of tumor suppressor gene products, which can be used therapeutically to block the export of tumor suppressor protein.

Additional compounds are contemplated in the invention method which modulate tumor suppressor function by interfering with or inhibiting the export of tumor suppressor protein. Such compounds either bind nuclear export signal and/or inhibit nuclear export signal function. Examples of compounds which are contemplated to modulate export of tumor suppressor protein include leptomycin A, CRM1, RanGTP, or fragments of CRM1 or RanGTP which bind nuclear export signal (see, e.g., Ossareh-Nazari et al. (1997) *Science* **278**:141-144), and the like.

The nuclear export signal of tumor suppressor proteins can be used to isolate proteins which interact with it. This interaction can be exploited to identify compounds which inhibit or interfere with this interaction. Inhibitors of protein-protein interactions between tumor suppressor proteins and other factors can lead to the development of pharmaceutical agents for the modulation of tumor suppressor activity. Methods to assay for protein-protein interactions, and to isolate proteins interacting with tumor suppressor protein are known to those skilled in the art.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

Creation of NES mutant constructs

Mutations were introduced into wild type p53 by two-step PCR mutagenesis with oligonucleotides containing point mutations corresponding to conversions of the leucine residues at 348 and/or 350 to alanines. For the double point mutant, the oligonucleotides 5'(1036) GAGGCCGCGGAAGCCAAGGATGCCCAGGCTGGG(1069) 3' (SEQ ID NO:1) and 5' (1056)ATCCTTGGCTTCCGCGGCCTCATTCAGCTCTCG(1024)3'(SEQ ID NO:2) were used to introduce the mutations and for the single mutant, the oligonucleotides 5'(1036)GAGGCCGCGGAACTCAAGGATGCCCAG GCTGGG(1069) 3' (SEQ ID

NO:3) and 5' (1056)ATCCTTGAGTTCCGCGGCCTCATTCAGCTCTCG (1024) 3' (SEQ ID NO:4) were used. The resultant mutated pieces were then coamplified with p53 coding sequence 5' and 3' primers with Sal I and Bam HI restriction sites on the ends to create a full length mutant construct without a stop codon. The PCR product was digested with Sal I and Bam HI and ligated into pEGFP-N1 (ClonTech) to create an in-frame C-terminal fusion with EGFP. The p53-GFP wild type and mutant constructs were subsequently subcloned into the pCLNCX retroviral construct (from Inder Verma of the Salk Institute) to create retrovirus.

Example 2

Electroporations

Four micrograms of each p53-GFP construct (wild type p53-GFP, 348-GFP, or 348/350-GFP constructs) were electroporated into Saos-2 ($p53^{-/-}$ osteosarcoma cells) or $p53^{-/-}$/$MDM2^{-/-}$ mouse embryonic fibroblasts at 0.25 kV and 960(F using a BioRad electroporator, plated on collagen-coated coverslips, and incubated 37°C, 7% CO_2 for 36 hours. Cells were washed in PBS, fixed in 3.7% formaldehyde for 10 minutes at room temperature, then stained with Texas Red-phalloidin (Molecular Probes) as per the manufacturers instructions. GFP and Texas Red staining was visualized on a Zeiss Axioplan 2 epifluorescence microscope using a 63x objective and images were captured using WinView/32 imaging software (Princeton Instruments) using 0.1-0.2 second exposure times for Texas Red and 1.0 to 4.0 second exposures for FITC. The wt p53-GFP construct was capable of cytoplasmic localization while the two NES mutants were exclusively nuclear in both cell lines.

Example 3

Glutaraldehyde crosslinking

N-terminal GST fusion constructs consisting of the tetramerization domain of p53 (amino acids 326-357), either wild type or 348/350 mutant, in pGEX2T (Pharmacia) were expressed in E. *colis* and purified and cleaved using Pharmacia's GST protein

Purification Module. Twenty micrograms of the cleaved tetramerization domain proteins were then incubated in 200 mM $NaPO_4$ pH 7.5 with or without 0.1 % glutaraldehyde (Sigma) at 37°C for thirty minutes, then run on a 20% SDS-PAGE gel and Coomassie Blue stained.

Example 4

Transfections

3.0×10^5 Saos-2 cells were co-transfected with 20, 100, or 500 ng of each p53-GFP construct, 750 ng of WWP-luciferase reporter (p21 promoter driving the expression of luciferase), and 700 ng of a lacz containing plasmid in 12-well plates using SuperFect reagent (Qiagen) as in the manufacturer's instructions, incubated for 48 hours, then assayed for luciferase and (-galactosidase activity.

Colony formation assay: 10^5 HS683 cells ($p53^{-/-}$ glioblastoma, obtained from Martin Haas) were infected 3 times over a sixteen hour period with wild type or mutant p53- GFP/pCLNCX retrovirus to a final multiplicity of infection of 2 or 10, then 24 hours later, split 1:2 into 75 cm^2 flasks. The following day the cells were treated with 0.8 mg/mL G418 and subsequently remained under selection for twenty days. The cells were then fixed for 10 minutes at room temperature with 3.7% formaldehyde, stained with crystal violet, and counted.

Example 5

Microinjection into HeLa cells

At least 24 hours prior to injection, HeLa cells grown in DMEM supplemented with 10% FCS were trypsinized, diluted 1:6 or 1:12 and seeded onto 10 mm diameter glass coverslips at 50% confluency. The coverslips were placed in a 3 cm dish containing CO_2-deficient medium (Gibco). Leptomycin B (LMB)-treated cells were cultured with 5 nM/ml LMB for 4 hrs before and during injection. Approximately 50 fl of a mixture of FITC labeled p53NES(wt)-BSA conjugates (~4 mg/ml) and Rhodamine-Dextran

(Sigma, 1.5 mg/ml) was injected into the nuclei of HeLa cells using an eppendorf microinjection system. In other experiments, an equal amount of FITC labeled p53NES(wt)-BSA and Rhodamine labeled p53NES(mut)-BSA were injected into the nuclei of HeLa cells. During injections, cells were incubated at 37° C utilizing a heated stage. After 4 hours of incubation at 37° C with normal DMEM 50%FCS in a CO_2 incubator the cells were fixed with 4% paraformaldehyde in PBS. Localization of the conjugates in the fixed cells was visualized by confocal microscopy.

Example 6

A highly conserved Rev-like nuclear export signal lies within the c-terminal tetramerization domain of p53

Because p53 had been previously shown to be cytoplasmically localized in both normal cycling cells and in many types of cancer cells, whether a nuclear export signal was involved in its subcellular localization was sought to be determined. Nuclear export has been shown to be mediated by nuclear export signals, leucine-rich peptide sequences first defined in HIV Rev and human PKI, which have the NES consensus sequence depicted in figure 1a. p53 has a leucine-rich sequence similar to the NES consensus motif in its c-terminus at amino acids 340-351 (fig. 1a). This sequence is highly conserved among the known sequenced p53s from humans to squid and is also present in the recently discovered p53 homologues p73 and KET. The p53 NES lies between two previously characterized nuclear localization signals found at amino acids 316-325 and 369-375 (figure 2). This sequence also lies within a key region of the tetramerization domain, the α-helix (amino acids 337-355) which is responsible for the formation of tetramers from pairs of dimers, and in fact the key hydrophobic residues of the putative NES are also crucial for tetramerization, L344, L348, and L350. This indicates a potential role for tetramerization in the nuclear export of p53.

To test whether the c-terminal Rev-like nuclear export signal consensus sequence mediates subcellular localization of p53, p53-GFP fusion constructs were created containing wild-type or two different mutant p53s containing either a single leucine-to-

alanine conversion at residue 348 or a double point mutant leucine-to-alanine at residues 348 and 350 (figure 3). Normal p53 function has been previously shown to be unaffected by the presence of a c-terminal GFP fusion in a variety of assays including transactivation of a p53 response element-containing reporter construct, inhibition of colony formation, and recognition by various protein-conformation specific antibodies. We also verified that our p53-GFP construct was able to activate transcription from a p21 promoter equivalently to unfused p53 (data not shown). The wild type and mutant p53-GFP constructs were either microinjected into the nuclei of the cells to be assayed or subcloned into a pCL M-MLV based retroviral packaging construct and subsequently used to create retrovirus to infect cells. Localization of the resultant fusion protein can then be determined by looking at GFP fluorescence under UV light. These approaches were adopted to avoid possible artifactual localization of p53 due to its response to cellular stress caused by more conventional methods such as calcium phosphate transfection.

Subcellular localization of wild type p53 varied but was consistent between the two methods of p53 introduction, with most cells having strong nuclear expression of p53-GFP and weaker cytoplasmic expression.. This variation was probably due to normal differential localization seen in a population of cells in different stages of the cell cycle. The NES mutants, however, consistently had strong nuclear expression and no cytoplasmic expression. These patterns of expression for the two constructs were consistent among many types of cells with variable endogenous p53 status, as summarized in table 1. Thus, the C-terminal rev-like NES consensus sequence of p53 confers its subcellular localization.

Example 7

The p53 NES is involved in nuclear-cytoplasmic shuttling

In order to verify that the exclusively nuclear localization of the p53 NES mutants was due to a defect in nuclear-cytoplasmic shuttling, ability of the wild type or NES mutant p53-GFP fusion constructs to be exported in a heterokaryon assay were tested.

$p53^{-/-}$/ $mdm2^{-/-}$ mouse embryonic fibroblasts were infected with either the wild type p53-GFP or the p53(348/350)-GFP containing retroviruses and incubated until GFP expression was visible under UV light. These cells were then mixed with uninfected human LFS041 fibroblasts which do not express endogenous p53, treated with cycloheximide to prevent *de novo* fusion protein synthesis, and fused. The heterokaryons were incubated {?}minutes after fusion to allow time for movement of the fusion proteins. If nuclear export is occurring, the fusion protein should shuttle out of the mouse nucleus, traverse the cytoplasm, and enter the human nucleus. Conversely, there should be no expression of the fusion protein in the human nucleus if it is unable to be exported from the mouse nucleus. Wild type p53-GFP is present in both the mouse and human nuclei and the cytoplasm, which can only occur if it had shuttled out of the mouse nucleus. p53(348/350)-GFP is only present in the mouse nucleus, indicating that mutating the NES impedes shuttling and thus this sequence mediates nuclear-cytoplasmic shuttling of p53.

A second experiment testing the ability of the p53 NES to mediate export of a carrier protein further demonstrated the necessity of the C-terminal NES for p53 shuttling. We created proteins consisting of a p53 peptide which spans the p53 NES (amino acids 326-357), either wild type or with the L348A and L350A NES point mutations , fused to the C-terminus of a carrier protein, glutathione S-transferase (GST) and purified them from *E. coli.* We then microinjected these fusion proteins into the nuclei of HeLa cells and assayed for movement of the constructs into the cytoplasm by fixing the cells and staining with an antibody to GST. Rhodamine-labeled dextran was coinjected to verify that the proteins were injected into the nucleus. Because GST self-associates into dimers, these fusion proteins would be too large to diffuse across the nuclear pore without active NES-mediated export. The wild type fusion GST-wt(326-357) moves from the nucleus into the cytoplasm, thus the NES of p53 is sufficient to mediate nuclear-cytoplasmic shuttling of the GST carrier protein. The double point mutant peptide fusion GST-348/350(326-357) was significantly impaired in its ability to shuttle as evidenced by its exclusively nuclear localization, demonstrating the necessity of the p53 NES consensus sequence for translocation across the nuclear pore.

As expected, unfused GST stayed exclusively nuclear throughout the course of the experiment.

Example 8

p53 is exported independently of hdm2

Because hdm2 has its own NES and is known to bind p53 and facilitate its degradation, its tempting to speculate that hdm2 binding is required for export of p53 from the nucleus. To assay the necessity of hdm2 for p53 export, we created p53/GFP fusion constructs containing mutations at amino acids 22 and 23 which are known to prevent binding of hdm2 to p53, then assayed for the localization of p53. 22/23-GFP (p53 mutant deficient for MDM2 binding) was electroporated into Saos-2 cells as in Example 2. 22/23-GFP or wt p53-GFP was electroporated into double knockout $p53^{-/-}$ /$MDM2^{-/-}$ mouse embryonic fibroblasts. Both MDM2 binding-deficient p53 in any genetic background and wild type p53 in $MDM2^{-/-}$ cells are capable of cytoplasmic localization, indicating that MDM2 is not required for the cytoplasmic localization of p53.

The hdm2-binding mutant is highly expressed in both the nucleus and the cytoplasm, indicating the existence of a mechanism of p53 export independent of binding by hdm2. The expression levels of these constructs are much higher than those of the wild type constructs because these are incapable of hdm2-mediated proteasomal degradation, verifying that these constructs are indeed unable to bind hdm2. The wild type p53-GFP fusions were also fully capable of cytoplasmic localization in a $p53^{-/-}$ / $mdm2^{-/-}$ mouse embryonic fibroblast cell line, further demonstrating the ability of p53 to be exported without the assistance of mdm2.

To verify that the hdm2 binding deficient p53 constructs are capable of nuclear-cytoplasmic shuttling despite their inability to bind hdm2, we assayed for shuttling in heterokaryons consisting of cycloheximide-treated $p53^{-/-}$ / $mdm2^{-/-}$ MEF cells infected with the p53(22/23)GFP retroviral construct fused to human LFS041 cells. Expression

of p53(22/23)GFP in the human nucleus indicates that the protein had to have been exported from the mouse nucleus, traveled through the cytoplasm, and imported into the human nucleus. This protein does indeed localize in the human nucleus, indicating that the protein was exported from the mouse nucleus, and that this export occurred independently of mdm2.

Example 9

The nuclear export of p53 utilizes a Rev-like NES pathway which includes CRM1

Recently it has been shown that the export of proteins containing a Rev-like nuclear export consensus sequence is mediated by the export-receptor protein, CRM1. This mechanism of export can be inhibited by the *Streptomyces* metabolite leptomycin B, an antibiotic known to bind CRM1 and prevent the formation of CRM1/RanGTP/NES complexes necessary for movement through the nuclear pore (Fornerod et al. (1997) supra). To determine if the NES of p53 utilizes this export pathway, we treated both human LFS041 cells and $p53^{-/-}$/ $mdm2^{-/-}$ MEF cells infected with either a wild type p53-GFP or a p53(348/350)-GFP retrovirus with leptomycin B, then fused them and assayed for movement of the GFP fusions between the mouse and human nuclei. If p53 does utilize this pathway, its export should be inhibited by the drug and no movement of the wild type fusion should take place, which is indeed what is observed. The NES mutant remains exclusively nuclear, since its export is prevented by mutations in the NES sequence which probably prevent its interaction with CRM1 irrespective of drug treatment.

In addition, wild type p53 was conjugated to FITC-labeled BSA with the crosslinking agent Sulfo-SMCC, then coinjected with Rhodamine-dextran into HeLa cells which had or had not been pre-treated with 5nM leptomycin B for 4 hous at 37°C. The injected cells were then allowed to incubate wih or without the drug an additional 4 hours. Leptomycin B is an inhibitor of nuclear export which prevents the export receptor, Exportin-1, from binding to an NES-containing protein. The p53 NES is

predominantly nuclear in the drug-treated cells, and therefor utilizes the Rev export pathway.

Example 10

p53 NES mutants are deficient in their ability to transactivate and form tetramers, but are still capable of growth suppression

It was hypothesized that a NES-mutant p53 would be more competent for transactivation and growth suppression than wild type p53 by virtue of its constitutively nuclear localization. To test this hypothesis, we transiently transfected Saos-2 (figure 5) and LFS041 cells with the p53-GFP fusion constructs used in the localization studies above and assayed the ability of these constructs to activate transcription from a p21 promoter. Both the double NES point mutant and the single point mutant were deficient in their transactivation ability relative to the wild type construct as measured by the induction of luciferase expression, although the single point mutant consistently had slightly higher activity. p53(22/23)-GFP is incapable of either transactivation or hdm2 binding and served as a negative control.

A possible explanation for this result is that because the p53 NES lies within and shares crucial residues with the tetramerization domain, perhaps the mutations introduced into the NES are affecting tetramerization as well. It has been shown previously that many p53-responsive promoters are only capable of being induced by tetrameric p53 and therefore would be incapable of being induced by a p53 mutant {{check on relative levels of tetramer vs. dimer}} deficient in tetramer formation. To test the hypothesis that our NES mutants are deficient in tetramer formation, we created the GST-wt(326-357) and GST-348/350(326-357) fusion peptides mentioned above plus a single point mutant peptide fusion GST-348(326-357). These peptides not only contain either a wild type or mutant NES but also the entire tetramerization domain of p53. Wild type or 348/350 mutant proteins consisting of the entire tetramerization domain were expressed in E. *coli*

as GST-fusions and subsequently cleaved off of the GST, at a fibronectin cleavage site between GST and the p53 peptide, which had been immobilized on a glutathione column.

Twenty micrograms of the cleaved proteins were then incubated at 37°C with or without increasing concentrations of 0.1% glutaraldehyde for 30 minutes, then run on a 20% SDS-PAGE gel, to covalently cross-link any dimers or tetramers that these constructs can allow to form. The wild type peptide is fully capable of forming tetramers and does so exclusively at the highest glutaraldehyde concentration. The double point mutant peptide, however, is significantly impaired in its ability to form higher order structures, and is only capable of forming dimers. Higher order structures created by the covalent crosslinking of monomeric subunits by glutaraldehyde are visible in both the wild type and NES mutant construct, however the wild type tetramerization domain is capable of forming both dimers and tetramers while the 348/350 mutant tetramerization domain can only form dimers and still retains a large quantity of monomer. This indicates that the crucial residues for NES function and tetramer formation are shared and hints that the regulation of nuclear export of p53 can be tied into the association or disassociation of p53 monomers.

Because it has been reported that p53 can be capable of transactivating some promoters and suppress colony formation as a dimer, we sought to determine the growth suppression activity of the dimeric NES mutants relative to that of the wild type tetramers. A colony formation assay is probably a better test of *in vivo* transcriptional activation potential than the transient transfection, because multiple genes are induced by p53 to cause growth suppression. Some of these genes can possibly be induced by dimeric as well as tetrameric p53. We infected two different glioblastoma cell lines, one p53 null and the other containing a DNA binding mutant p53, with each of the four different p53-GFP retroviral constructs, wt p53-GFP, p53(22/23)-GFP, p53(348)-GFP, and p53(348/350)-GFP. The infected cells were subjected to selection for retroviral gene expression with geneticin and incubated 10-20 days until colonies formed. Because the p53(22/23)-GFP is deficient in DNA binding, we see that it is vastly deficient in growth suppression relative to wt p53-GFP (figure 6). However, we see that the NES mutants,

in spite of their inability to form tetramers, were still capable of inhibiting colony formation.

Example 11

The p53 NES can export a heterologous protein

Wild type or 348/350 mutant peptide consisting of amino acids 339-352 containing the NES were covalently conjugated to FITC or Rhodamine-labeled BSA with the crosslinking reagent Sulfo-SMCC. Wild type NES BSA-FITC was then coinjected with either Rhodamine-dextran (a marker for localization of injections, >40kDa) or mutant NES BSA-Rhodamine into the nuclei of HeLa cells, and incubated for 4 hrs. at 37°C to allow sufficient time for export. The wild type p53 NES can facilitate the export of BSA (68kDa) while mutated while mutated p53 NES can only do so inefficiently.

Example 12

Method to identify hydrophobic or Rev-like (CRM1 interacting) nuclear export signals in tumor suppressor genes

Often, leucines are found in the four key positions of NESs. However, any large hydrophobic amino acid can occupy a key position. The following amino acids can be involved in NES function:

Leucine (L)
Isoleucine (I)
Valine (V)
Methionine (M)
Cystine (C)
Tryptophan (W)
Phenylalanine (F)

The Key to NES function is the spacing of these large hydrophobic

The NESs can be divided into two classes. The Typical NESs contain a core tetramer (LxLy) motif. The L represent positions which must be occupied by a large hydrophobic amino acid. The x is a highly variable sequence, while the y tends to be a charged amino acid (K, R, E, D), Q is also common.

After identifying core tetramers, the next step is to look for two hydrophobic amino acids located upstream of the core tetramer. The positioning of the upstream hydrophobic amino acids is variable. The first hydrophobic amino acid must be located 3 or 4 amino acids upstream of the core tetramer. The second hydrophobic amino acid is located 3 or 4 amino acids upstream of the first variable hydrophobic. Thus, the variable upstream hydrophobic amino acids can have the following combinations relative to the core tetramer: -8,-4; -7,-4; ,-7,-3; or -6.-3. For simplicity, the positions which must be occupied by hydrophobic amino acids are represented by L (leucine). The positioning of the variable hydrophobics is shown below:

8 7 6 5 4 3 2 1 L x L y

-8,-4; L L L x L y
-7,-4; L L L x L y
-7,-3; L L L x L y
-6,-3 L L L x L y

An additional characteristic is that there are often a number of charged amino acids (K, R, D, E) located at other positions throughout the NESs. It is proposed that this is important for keeping the NES sequence on the surface of the protein. Prolines can also be found within CRM1 mediated NESs. The experiments disclosed herein also reveal that it is possible to mutate one or more of the two upstream hydrophobic amino acids and still retain some NES activity in certain contexts.

There is a subclass of the typical Rev-like (CRM1 mediated) NESs that consist of two copies of the core tetramer (as defined above) separated by a single amino acid. The structure of a "double core" NES is shown below:

L x L y x L x L y (SEQ ID NO:5)

Finally, there are several known CRM1 mediated NESs which have an atypical spacing of four required amino acids. These NESs, known as the atypical NESs, do not contain a core tetramer. The NESs in the Rev proteins of equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) contain atypical NESs. Shown below is the example of the atypical NES of EIAV.

L E S D Q W C R V L R Q S L (SEQ ID NO:6)

Our studies reveal that the underlined hydrophobic amino acids are required for function. The approaches designated her are designed to identify putative NESs. However, the functionality of each putative sequence must be determined experimentally. Additionally, a protein can contain more than one NES.

Following is a example of using the above described technique to identify putative CRM1 mediated NESs in the tumor suppressor genes RB, BRCA1, and BRCA2. To facilitate viewing of the spacing of hydrophobic amino acids, the amino acids L, I, V, M, C, F, W are bolded. Putative NESs are then underlined. Putative "double core" NESs are shown with a double underline.

HUMAN RB (SEQ ID NO:7)

MPPKTPRKTAATAAAAAAEPPAPPPPPPEEDPEQDSGPEDLPLVRLEFEETE
EPDFTALCQKLKIPDHVRERAWLTWEKVSSVDGVLGGYIQKKKELWGICIF
IAAVDLDEMSFTFTELQKNIEISVHKFFNLLKEIDTSTKVDNAMSRLLKKYD
VLFALFSKLERTCELIYLTQPSSSISTEINSALVLKVSWITFLLAKGEVLQME

DDLVISFQLMLCVLDYFIKLSPPMLLKEPYKTAVIPINGSPRTPRRGQNRSAR
IAKQLENDTRIIEVLCKEHECNIDEVKNVYFKNFIPFMNSLGLVTSNGLPEVE
NLSKRYEEIYLKNKDLDARLFLDHDKTLQTDSIDSFETQRTPRKSNLDEEVN
VIPPHTPVRTVMNTIQQLMMILNSASDQPSENLISYFNNCTVNPKESILKRVK
DIGYIFKEKFAKAVGQGCVEIGSQRYKLGVRLYYRVMESMLKSEEERLSIQ
NFSKLLNDNIFHMSLLACALEVVMATYSRSTSQNLDSGTDLSFPWILNVLN
LKAFDFYKVIESFIKAEGNLTREMIKHLERCEHRIMESLAWLSDSPLFDLIK
QSKDREGPTDHLESACPLNLPLQNNHTAADMYLSPVRSPKKKGSTTRVNST
ANAETQATSAFQTQKPLKSTSLSLFYKKVYRLAYLRLNTLCERLLSEHPELE
HIIWTLFQHTLQNEYELMRDRHLDQIMMCSMYGICKVKNIDLKFKIIVTAY
KDLPHAVQETFKRVLIKEEEYDSIIVFYNSVFMQRLKTNILQYASTRPPTLSPI
PHIPRSPYKFPSSPLRIPGGNIYISPLKSPYKISEGLPTPTKMTPRSRILVSIGESF
GTSEKFQKINQMVCNSDRVLKRSAEGSNPPKPLKKLRFDIEGSDEADGSKHL
PGESKFQQKLAEMTSTRTRMQKQKMNDSMDTSNKEEK

HUMAN BRCA1 (SEQ ID NO:8)

MDLSALRVEEVQNVINAMQKILECPICLELIKEPVSTKCDHIFCKFCMLKLL
NQKKGPSQCPLCKNDITKRSLQESTRFSQLVEELLKIICAFQLDTGLEYANS
YNFAKKENNSPEHLKDEVSIIQSMGYRNRAKRLLQSEPENPSLQETSLSVQL
SNLGTVRTLRTKQRIQPQKTSVYIELGSDSSEDTVNKATYCSVGDQELLQITP
QGTRDEISLDSAKKAACEFSETDVTNTEHHQPSNNDLNTTEKRAAERHPEKY
QGSSVSNLHVEPCGTNTHASSLQHENSSLLLTKDRMNVEKAEFCNKSKQPG
LARSQHNRWAGSKETCNDRRTPSTEKKVDLNADPLCERKEWNKQKLPCSE
NPRDTEDVPWITLNSSIQKVNEWFSRSDELLGSDDSHDGESESNAKVADVLD
VLNEVDEYSGSSEKIDLLASDPHEALICKSERVHSKSVESNIEDKIFGKTYRK
KASLPNLSHVTENLIIGAFVTEPQIIQERPLTNKLKRKRRPTSGLHPEDFIKKA
DLAVQKTPEMINQGTNQTEQNGQVMNITNSGHENKTKGDSIQNEKNPNPIES
LEKESAFKTKAEPISSSISNMELELNIHNSKAPKKNRLRRKSSTRHIHALELV
VSRNLSPPNCTELQIDSCSSSEEIKKKKYNQMPVRHSRNLQLMEGKEPATGA
KKSNKPNEQTSKRHDSDTFPELKLTNAPGSFTKCSNTSELKEFVNPSLPREEK

EEKLETVKVSNNAEDPKDLMLSGERVLQTERSVESSSISLVPGTDYGTQESIS
LLEVSTLGKAKTEPNKCVSQCAAFENPKGLIHGCSKDNRNDTEGFKYPLGH
EVNHSRETSIEMEESELDAQYLQNTFKVSKRQSFALFSNPGNAEEECATFSA
HSGSLKKQSPKVTFECEQKEENQGKNESNIKPVQTVNITAGFPVVGQKDKPV
DNAKCSIKGGSRFCLSSQFRGNETGLITPNKHGLLQNPYRIPPLFPIKSFVKT
KCKKNLLEENFEEHSMSPEREMGNENIPSTVSTISRNNIRENVFKGASSSNIN
EVGSSTNEVGSSINEIGSSDENIQAELGRNRGPKLNAMLRLGVLQPEVYKQS
LPGSNCKHPEIKKQEYEEVVQTVNTDFSPYLISDNLEQPMGSSHASQVCSETP
DDLLDDGEIKEDTSFAENDIKESSAVFSKSVQRGELSRSPSPFTHTHLAQGYR
RGAKKLESSEENLSSEDEELPCFQHLLFGKVNNIPSQSTRHSTVATECLSKNT
EENLLSLKNSLNDCSNQVILAKASQEHHLSEETKCSASLFSSQCSELEDLTA
NTNTQDPFLIGSSKQMRHQSESQGVGLSDKELVSDDEERGTGLEENNQEEQS
MDSNLGEAASGCESETSVSEDCSGLSSQSDILTTQQRDTMQHNLIKLQQEM
AELEAVLEQHGSQPSNSYPSIISDSSALEDLRNPEQSTSEKAVLTSQKSSEYPI
SQNPEGLSADKFEVSADSSTSKNKEPGVERSSPSKCPSLDDRWYMHSCSGSL
QNRNYPSQEELIKVVDVEEQQLEESGPHDLTETSYLPRQDLEGTPYLESGISL
FSDDPESDPSEDRAPESARVGNIPSSTSALKVPQLKVAESAQGPAAAHTTDTA
GYNAMEESVSREKPELTASTERVNKRMSMVVSGLTPEEFMLVYKFARKHHI
TLTNLITEETTHVVMKTDAEFVCERTLKYFLGIAGGKWVVSYFWVTQSIKE
RKMLNEHDFEVRGDVVNGRNHQGPKRARESQDRKIFRGLEICCYGPFTNMP
TDQLEWMVQLCGASVVKELSSFTLGTGVHPIVVVQPDAWTEDNGFHAIGQ
MCEAPVVTREWVLDSVALYQCQELDTYLIPQIPHSHY

HUMAN BRCA2 (SEQ ID NO:9)

MPVEYKRRPTFWEIFKARCSTADLGPISLNWFEELSSEAPPYNFEPPEESEYK
PHGYEPQLFKTPQRNPPYHQFASTPIMFKERSQTLPLDQSPFRELGKVVASSK
HKTHSKKKTKVDPVVDVASPPLKSCLSESPLTLRCTQAVLQREKPVVSGSLF
YTPKLKEGQTPKPISESLGVEVDPDMSWTSSLATPPTLSSTVLIARDEEARSS
VTPADSPATLKSCFSNHNESPQKNDRSVPSVIDSENKNQQEAFSQGLGKMLG
DSSGKRNSFKDCLRKPIPNILEDGETAVDTSEEDSFSLCFPKRRTRNLQKMR

MGKTRKKIFSETRTDELSEEARRQPDDKNSFVFEMELRESDPLDPGVTSQKP
FYSQNEEICNEAVQCSDSRWSQSNLSGLNETQTGKITLPHISSHSQNISEDFID
MKKEGTGSITSEKSLPHISSLPEPEKMFSEETVVDKEHEGQHFESLEDSIAGK
QMVSRTSQAACLSPSIRKSIFKMREPLDETLGTVFSDSMTNSTFTEEHEASAC
GLGILTACSQREDSICPSSVDTGSWPTTLTDTSATVKNAGLISTLKNKKRKFI
YSVSDDASLQGKKLQTHRQLELTNLSAQLEASAFEVPLTFTNVNSGIPDSSD
KKRCLPNDPEEPSLTNSFGTATSKEISYIHALISQDLNDKEAIVIEEKPQPYTA
READFLLCLPERTCENDQKSPKVSNGKEKVLVSACLPSAVQLSSISFESQENP
LGDHNGTSTLKLTPSSKLPLSKADMVSREKMCKMPEKLQCESCKVNIELSK
NILEVNEICILSENSKTPGLLPPGENIIEVASSMKSQFNQNAKIVIQKDQKGSP
FISEVAVNMNSEELFPDSGNNFAFQVTNKCNKPDLGSSVELQEEDLSHTQGP
SLKNSPMAVDEDVDDAHAAQVLITKDSDSLAVVHDYTEKSRNNIEQHQKGT
EDKDFKSNSSLNMKSDGNSDCSDKWSEFLDPVLNHNFGGSFRTASNKEIKL
SEHNVKKSKMFFKDIEEQYPTRLACIDIVNTLPLANQKKLSEPHIFDLKSVTT
VSTQSHNQSSVSHEDTDTAPQMLSSKQDFHSNNLTTSQKAEITELSTILEESG
SQFEFTQFRKPSHIAQNTSEVPGNQMVVLSTASKEWKDTDLHLPVDPSVGQ
TDHSKQFEGSAGVKQSFPHLLEDTCNKNTSCFLPNINEMEFGGFCSALGTKL
SVSNEALRKAMKLFSDIENSEEPSAKVGPRGFSSSAHHDSVASVFKIKKQNT
EKSFDEKSSKCQVTLQNNIEMTTCIFVGRNPEKYIKNTKHEDSYTSSQRNNL
ENSDGSMSSTSGPVYIHKGDSDLPADQGSKCPESCTQYAREENTQIKENISDL
TCLEIMKAEETCMKSSDKKQLPSDKMEQNIKEFNISFQTASGKNTRVSKESL
NKSVNIFNRETDELTVISDSLNSKILHGINKDKMHTSCHKKAISIKKVFEDHF
PIVTVSQLPAQQHPEYEIESTKEPTLLSFHTASGKKVKIMQESLDKVKNLFDE
TQYVRKTASFSQGSKPLKDSKKELTLAYEKIEVTASKCEEMQNFVSKETEM
LPQQNYHMYRQTENLKTSNGTSSKVQENIENNVEKNPRICCICQSSYPVTED
SALAYYTEDSRKTCVRESSLSKGRKWLREQGDKLGTRNTIKIECVKEHTEDF
AGNASYEHSLVIIRTEIDTNHVSENQVSTLLSDPNVCHSYLSQSSFCHCDDM
HNDSGYFLKNKIDSDVPPDMRNAEGNTISPRVSATKERNLHPQTINEYCVQK
LETNTSPHANKDVAIDPFLLDSRNCKVGSLVFITAHSQETERTKEIVTDNCY
KIVEQNRQSKPDTCQTSCHKVLDDSKDFICPSSSGDVCINSRKDSFCPHNEQI
LQHNQSMFGLKKAATPPVGLETWDTSKSIREPPQAAHPSRTYGIFSTASGKA

IQVSDASLEKARQVFSEMDGDAKQLSSMVSLEGNEKPHHSVKRENSVVHST
QGVLSLPKPLPGNVNSSVFSGFSTAGGKLVTVSESALHKVKGMLEEFDLIRT
EHTLQHSPIPEDVSKILPQPCAEIRTPEYPVNSKLQKTYNDKSSLPSNYKESGS
SGNTQSIEVSLQLSQMERNQDTQLVLGTKVSHSKANLLGKEQTLPQNIKVK
TDEMKTFSDVPVKTNVGEYYSKESENYFETEAVESAKAFMEDDELTDSEQT
HAKCSLFTCPQNETLFNSRTRKRGGVTVDAVGQPPIKRSLLNEFDRIIESKGK
SLTPSKSTPDGTVKDRSLFTHHMSLEPVTCGPFCSSKERQGAQRPHLTSPAQE
LLSKGHPWRHSALEKSPSSPIVSILPAHDVSATRTERTRHSGKSTKVFVPPFK
MKSQFHGDEHFNSKNVNLEGKNQKSTDGDREDGNDSHVRQFNKDLMSSLQ
SARDLQDMRIKNKERRHLRLQPGSLYLTKSSTLPRISLQAAVGDRAPSACSP
KQLYIYGVSKECINVNSKNAEYFQFDIQDHFGKEDLCAGKGFQLADGGWLI
PSNDGKAGKEEFYRALCDTPGVDPKLISSIWVANHYRWIVWKLAAMEFAF
PKEFANRCLNPERVLLQLKYRYDVEIDNSRRSALKKILERDDTAAKTLVLCI
SDIISPSTKVSETSGGKTSGEDANKVDTIELTDGWYAVRAQLDPPLMALVKS
GKLTVGQKIITQGAELVGSPDACAPLEAPDSLRLKISANSTRPARWHSRLGF
FCDPRPFPLPLSSMFSDGGNVGCVDIIVQRVYPLQWVEKTVSGLYIFRSERE
EEKEALRFAEAQQKKLEALFTKVHTEFKDHEEDTTQRCVLSRTLTRQQVHA
LQDGAELYAAVQYASDPDHLEACFSEEQLRALNNYRQMLNDKKQARIQSE
FRKALESAEKEEGLSRDVTTVWKLRVTSYKKKEKSALLIIWRPSSDLSSLLT
EGKRYRIYLLAVSKSKSKFERPSIQLTATKRTQYQQLPVSSETLLQVYQPRES
LHFSRLSDPAFQPPCSEVDVVGVVVSVVKPIGLAPLVYLSDECLNLLVVKFG
IDLNEDIKPRVLIAASNLQCQPESTSGVPTLFAGHFSIFSASPKEAYFQEKVN
NLKHAIENIDTFYKEAEKKLIHVLEGDSPKWSTPNKDPTREPHAASTCCASD
LLGSGGQFLRISPTGQQSYQSPLSHCTLKGKSMPLAHSAQMAAKSWSGENE
IDDPKTCRKRRALDFLSRLPLPSPVSPICTFVSPAAQKGFQPPRSCGTKYATPI
KKKPSSPRRKTPFQKTSGVSLPDCDSVADEELALLSTQALTPDSVGGNEQAF
PGDSTRNPQPAQRPDQQVGPRSRKESLRDCRGDSSEKLAVES

Example 13

Demonstrating functional nature of NESs

The functional nature of NESs should be demonstrated in two ways. This will demonstrate that the putative NES can infact function as an export signal in a heterologous context, and that the sequence functions as a NES in the proeinm in question.

First: Demonstrate that the putative NES can function as an NES using in vivo assays.

A) Rev complementation assay.

To determine if a putative NES is functional, it is possible to utilize a complementation assay based on the HIV Rev protein. Rev requires a NES to perform its function of exporting unspliced RNA out of the nucleus. We have previously shown that the function of a NES-mutated Rev can be restored by the fusion of a heterologous NES. A related complementation assay has been utilized to identify the NES in the transcription factor TFIIIA and the Fragile-X Mental Retardation Protein (FxMRP1). However, a functional NES may not complement Rev function due to protein folding problems. For this reason a negative result with this assay does not exclude the possibility that the putative sequence is indeed a NES.

B) Nuclear microinjection assay.

The most direct demonstration of NES function is a nuclear microinjection assay. In this assay a recombinant proteins such as glutathione-s-transferase (GST) fused to the region of interest is purified and injected into the nucleus of a fibroblast cell, such as Cos7 or 3T3, along with an injection marker such as fluorescently labeled dextran, which is too large to diffuse through the nuclear pore. An alternative approach is to synthesize a peptide encoding the putative NES. Conjugate the peptide to BSA. Fluorescently label the BSA-peptide conjugates and microinject them into cells. If the peptide conjugates or fusion proteins export from the nucleus, the signal has the potential to be a NES.

Second: Mutation of the NES within the protein perturbs the normal biology of that protein.

If the identified region is a NES, mutation of this sequence in the context of the full protein should perturb the normal localization of the test protein, or the ability to the test protein shuttle between the nuclear and cytoplasmic compartments. Mutation of the two hydrophobic amino acids within the core tetramer should disrupt NES function.

If the protein is normally localized within the cytoplasm, or throughout the cell, mutation of the NES will shift localization to the nucleus. If the protein is normally nuclear, mutation of the NES will block the shuttling of the protein. The shuttling of a protein with a steady state nuclear localization is best monitored with a heterokaryon assay. In the heterokaryon assay, the protein of interest is expressed in a mouse derived fibroblast cell line. Human fibroblast cells are added to the culture. After the cells have adhered, the culture is treated with cyclohexamide for 30 minutes and the cells are fused by treatment with polyethylene glycol. After 4 hours, the cells are fixed and identified by immunofluorescent analysis. The nuclei of mouse and human cells are differentially stained with Hoescht dye. The mouse nuclei will contain easily recognizable speckles when stained with this dye. The presence of the protein of interest in human nuclei indicate that the protein of interest is shuttling between the nucleus and cytoplasm. Cyclohexamide treatment insures that any protein of interest in the human nucleus must have come from preexisting protein in the mouse nucleus. Once the protein of interest exports from the mouse nuclei, it has a equal possibility of reimporting into either type of nucleus. If the identified sequence is an NES, it should prevent the shuttling of the protein of interest. It must also be remembered that the protein of interest can have more than one NES. One method to determine this possibility is to determine the effect of leptomycin B (LMB) treatment on protein localization and shuttling. LMB blocks the function of CRM1, the export receptor for the Rev-like leucine-rich NESs. Therefore, LMB treatment will perturb protein localization as described above for mutation of the NES.

Example 14

Generation of BSA-peptide conjugates

Two peptides with the following sequence were utilized in this study, p53NES(wt): CEMFRELNEALELKD (SEQ ID NO:10), p53NES(mut): CEMFRELNEAAEAKD (SEQ ID NO:11). BSA-peptide conjugates were generated using a modification of the protocol of Fischer et. al. Six ml of BSA (5mg/ml) (Boeringer Mannheim) in phosphate buffered saline (PBS) (pH 7.4) was incubated with 50 mg of sulfosuccinmidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce Chemical) for 1 hr at room temperature. The BSA was subsequently separated from the unreacted sulfo-SMCC with chromatography over G-50 sephadex (fine) (Pharmecia). Approximately five milligrams of the purified sulfo-SMCC BSA (1ml) was mixed with 1 ml of PBS (pH 6.5) containing 40% dimethyl-sulfoxide (DMSO) and incubated with 10 mg of peptide overnight at 4 C. BSA-peptide conjugated were then separated from free peptides and DMSO gel filtration chromatography (G-50 sephadex) Conjugation was assessed by SDS-PAGE. The altered mobility revealed that each BSA molecule contained approximately 15-20 peptides. The altered mobility was similar for both peptides. Only a small fraction of crosslinked BSA was detected. An aliquot of each derivative was labeled with FITC-maleimide (Pierce Chemical), Rhodamine Red C_2 maleimide (Molecular Probes) in PBS (pH 7.4), 50% DMSO for 2 hrs at room temperature. The resulting labeled BSA-peptide conjugate was then separated from unincorporated label by G-50 Sephadex (equilibrated with PBS, pH 7.4) chromatography followed by concentration by centrifugation in a Centricon C-50 unit (Amicon).

That which is claimed is:

1. A peptide having tumor suppressor function, wherein said peptide comprises a tumor suppressor protein having impeded nuclear export function.

2. A peptide according to claim 1, wherein said peptide is unable to associate with a component of the nuclear export machinery.

3. A peptide according to claim 2, wherein said peptide further comprises a nuclear export signal inhibited from associating with said component.

4. A peptide according to claim 1, wherein said tumor suppressor protein is selected from p53, p73, KET, RB1, WT1, NF1, NF2, VHL, APC, DCC, NB1, MLM, MEN1, MEN2A, BCNS, RCC, BRCA1, BRCA2 or ETS1.

5. A peptide according to claim 1, wherein export of said peptide from a cell nucleus is impeded by alteration of a nuclear export signal of said tumor suppressor protein.

6. A peptide according to claim 5, wherein said nuclear export signal is altered by amino acid substitution, deletion, addition and/or modification.

7. A peptide according to claim 6, wherein at least one hydrophobic amino acid residue within the nuclear export signal of a tumor suppressor gene is substituted, deleted and/or modified.

8. A peptide according to claim 2, wherein said peptide further comprises an oligomerization domain which lacks export function.

9. A peptide according to claim 2, wherein said peptide is further altered to prevent interaction with negative regulator(s).

10. A nucleic acid encoding a modified peptide according to claim 1.

11. An expression vector comprising the nucleic acid of claim 11 operatively associated with a regulatory sequence.

12. A method for treating a neoplastic condition in a subject in need thereof, said method comprising administering to said subject an effective amount of a modified peptide according to claim 1, or nucleic acid encoding same.

13. A method according to claim 12, wherein said neoplastic condition is selected from the group consisting of breast carcinoma, ovarian carcinoma, sarcomas, gliomas, retinoblastomas, Wilm's tumor, schwannoma, pheochromocytoma, kidney carcinoma, neuroblastoma, melanoma, pituitary adenoma, and medulloblastoma.

14. A method according to claim 13, wherein said gliomas are treated by administering a modified p53 or NF1 peptide, or nucleic acid encoding same.

15. A method according to claim 13, wherein said breast carcinoma is treated by administering a modified p53, BRCA1 or BRCA2 peptide, or nucleic acid encoding same.

16. A method according to claim 13, wherein said ovarian carcinoma is treated by administering a modified p53 peptide, or nucleic acid encoding same.

17. A method according to claim 13, wherein said sarcomas are treated by administering a modified p53, NF1 or RB1 peptide, or nucleic acid encoding same.

18. A method according to claim 13, wherein said retinoblastoma is treated by administering a modified RB1 peptide, or nucleic acid encoding same.

19. A method according to claim 13, wherein said Wilm's tumor is treated by administering a modified WT1 peptide, or nucleic acid encoding same.

20. A method according to claim 13, wherein said schwannoma is treated by administering a modified VHL peptide, or nucleic acid encoding same.

21. A method according to claim 13, wherein said pheochromocytoma is treated by administering a modified APC peptide, or nucleic acid encoding same.

22. A method according to claim 13, wherein said kidney carcinoma is treated by administering a modified APC or RCC peptide, or nucleic acid encoding same.

23. A method according to claim 13, wherein said neuroblastoma is treated by administering a modified NB1 or p73 peptide, or nucleic acid encoding same.

24. A method according to claim 13, wherein said melanoma is treated by administering a modified MLM peptide, or nucleic acid encoding same.

25. A method according to claim 13, wherein said pituitary adenoma is treated by administering a modified MEN1 peptide, or nucleic acid encoding same.

26. A method according to claim 13, wherein said medulloblastoma is treated by administering a modified BCNS peptide, or nucleic acid encoding same.

27. A method according to claim 13, wherein said skin carcinoma is treated by administering a modified BCNS peptide, or nucleic acid encoding same.

28. A method according to claim 12 wherein said peptide is supplied by administering nucleic acid encoding same to said subject.

29. A method according to claim 12 wherein nucleic acid encoding said peptide is supplied ex vivo to cells of said subject.

p53 contains a highly-conserved, rev-like NES

Rev-like consensus:			Z	X_{2-3}	Z	X_{2-3}	L/V	X	L/I	X	
Published NESs:	HIV rev:		L	P	P	L	E	R	L	T	L	D
	PKI, human:		L	A	L	K	L	AG	L	D	I	N
	MDM2:		I	S	L	S	F	DES	L	A	L	C
p53 NESs:	human:	340	M	F	R	E	L	NEA	L	E	L	K	351
	mouse:	337	M	F	R	E	L	NEA	L	E	L	K	348
	rabbit:		M	F	R	E	L	NEA	L	E	L	K	
	cow:		M	F	R	E	L	NDA	L	E	L	K	
	Xennopus:		M	I	K	K	L	NDA	L	E	L	Q	
	zebrafish:		I	L	K	K	L	NDS	L	E	L	S	
	squid:		I	L	C	K	L	RDI	M	E	L	D	
p53 family member NESs (putative):	p73:		I	L	M	K	L	KES	L	E	L	M	
	KET:		M	L	L	K	I	KES	L	E	L	M	

Figure 1

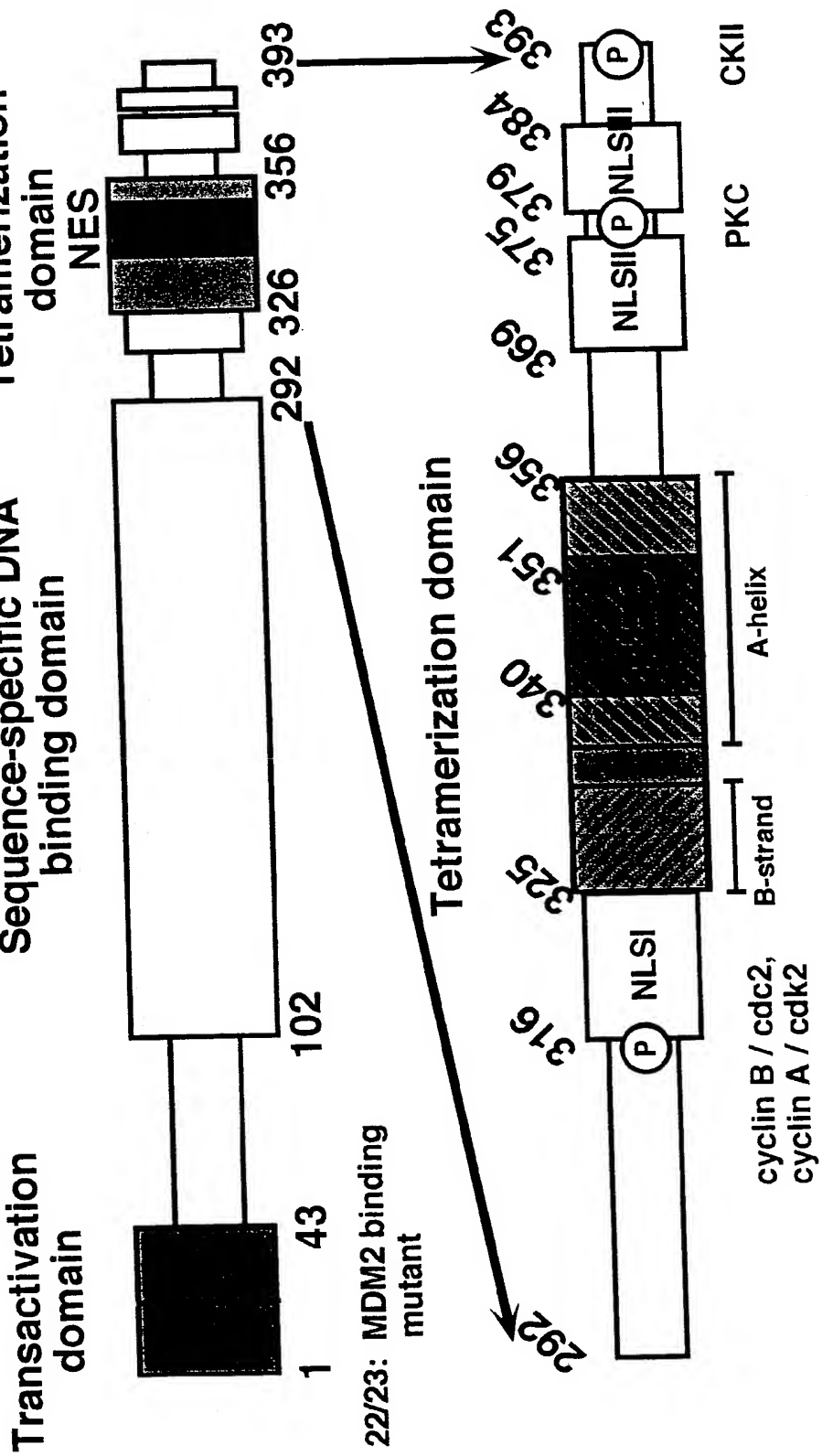
p53 NES CONTEXT WITHIN THE C-TERMINUS
Transactivation domain
Sequence-specific DNA binding domain
Tetramerization domain
NES
Tetramerization domain
NLSI
NLSII
NLSIII
PKC
CKII
A-helix
B-strand
cyclin B / cdc2, cyclin A / cdk2
22/23: MDM2 binding mutant
1
43
102
292
326
356
393
316
325
340
351
356
369
375
379
384
393
292

Figure 2

p53 NES mutants:

wt: 340 - MFRELNEALELK - 351
348: 340 - MFRELNEAAELK - 351
348/350: 340 - MFRELNEAAEAK - 351

Figure 3

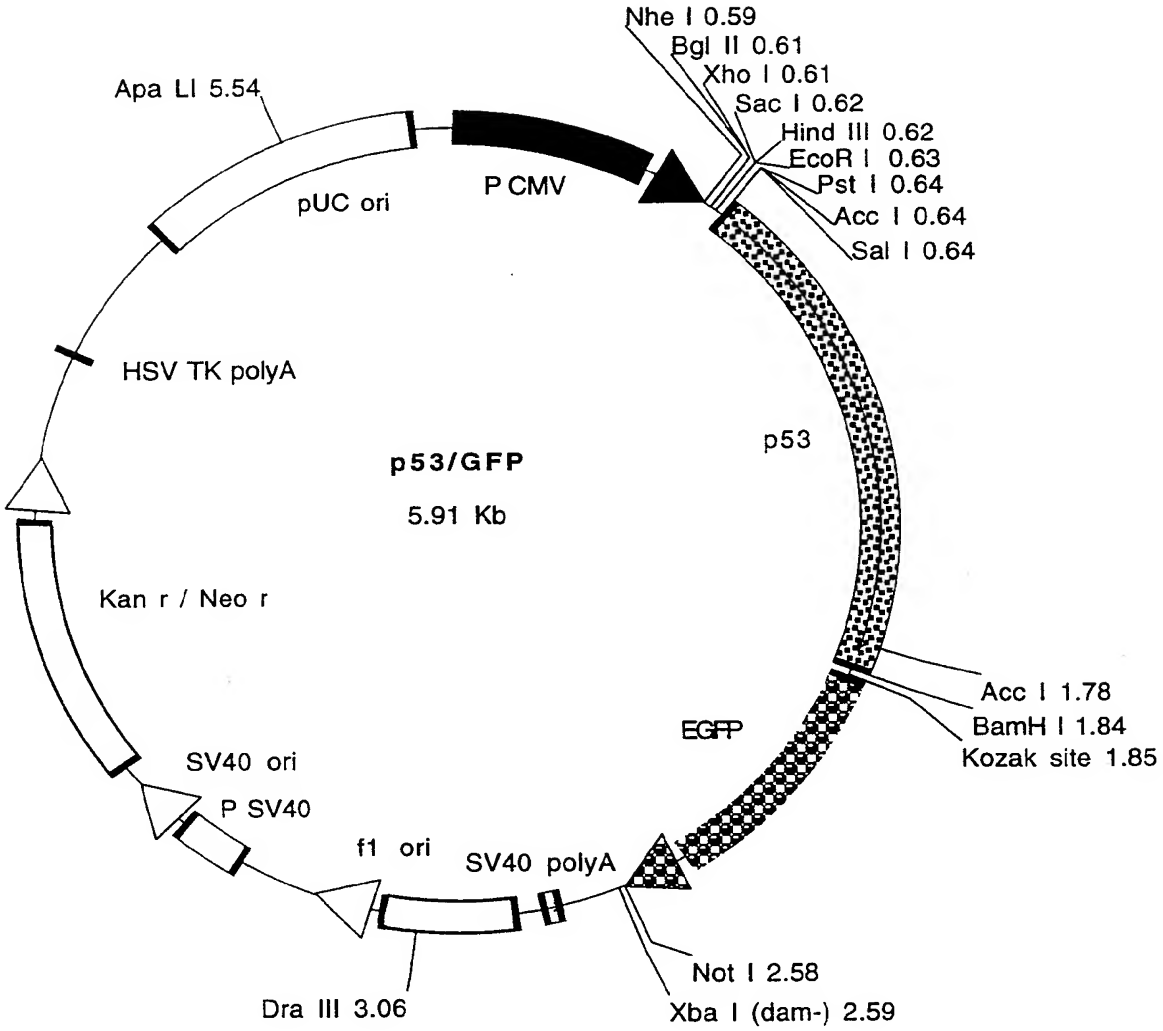
Nhe I 0.59
Bgl II 0.61
Xho I 0.61
Sac I 0.62
Hind III 0.62
EcoR I 0.63
Pst I 0.64
Acc I 0.64
Sal I 0.64
Apa LI 5.54
pUC ori
P CMV
HSV TK polyA
p53
p53/GFP
5.91 Kb
Kan r / Neo r
Acc I 1.78
BamH I 1.84
Kozak site 1.85
EGFP
SV40 ori
P SV40
f1 ori
SV40 polyA
Not I 2.58
Xba I (dam-) 2.59
Dra III 3.06

Figure 4

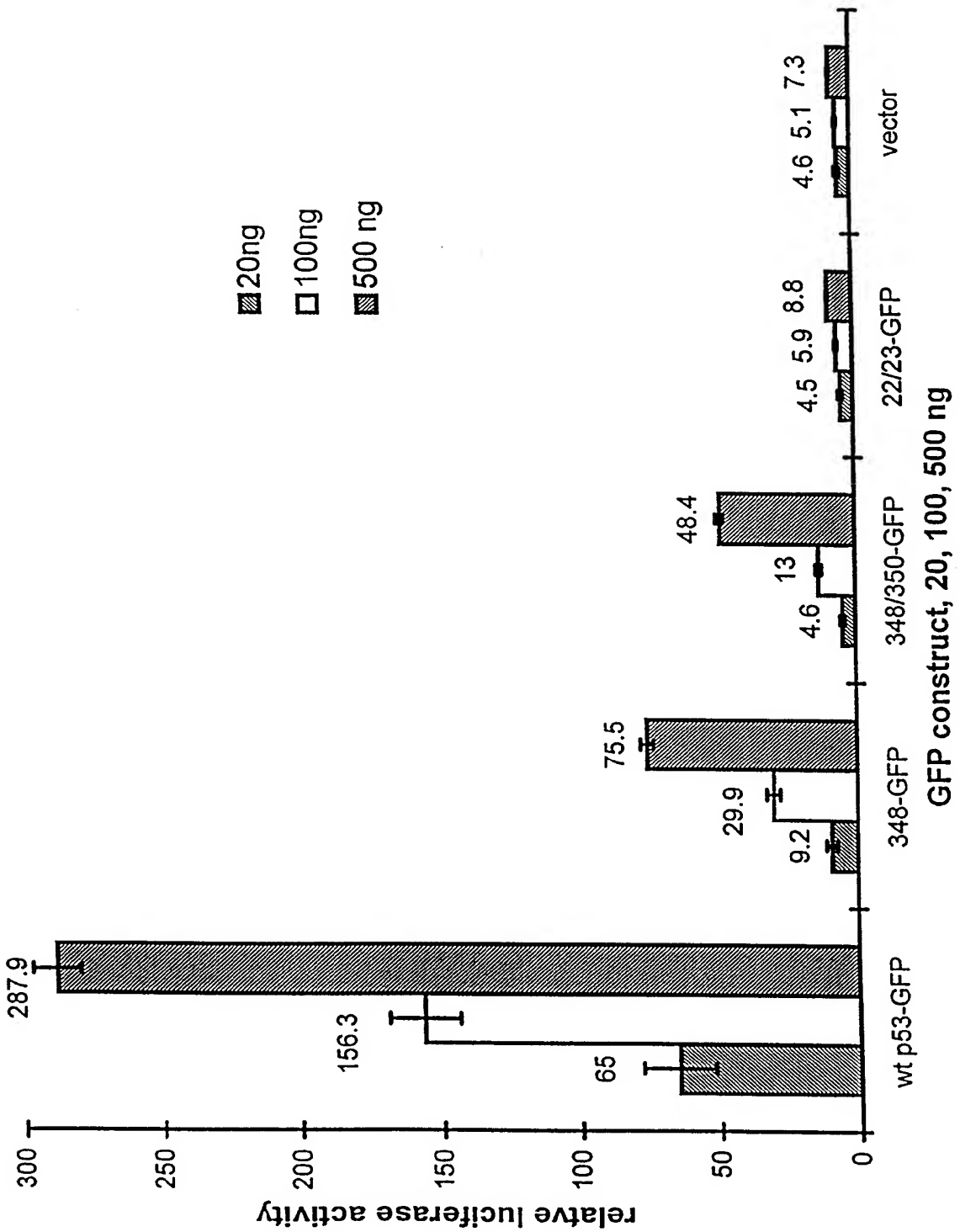
20ng
100ng
500 ng
relative luciferase activity
300
250
200
150
100
50
0
287.9
156.3
65
75.5
29.9
9.2
48.4
13
4.6
4.5 5.9 8.8
4.6 5.1 7.3
wt p53-GFP
348-GFP
348/350-GFP
22/23-GFP
vector
GFP construct, 20, 100, 500 ng

Figure 5

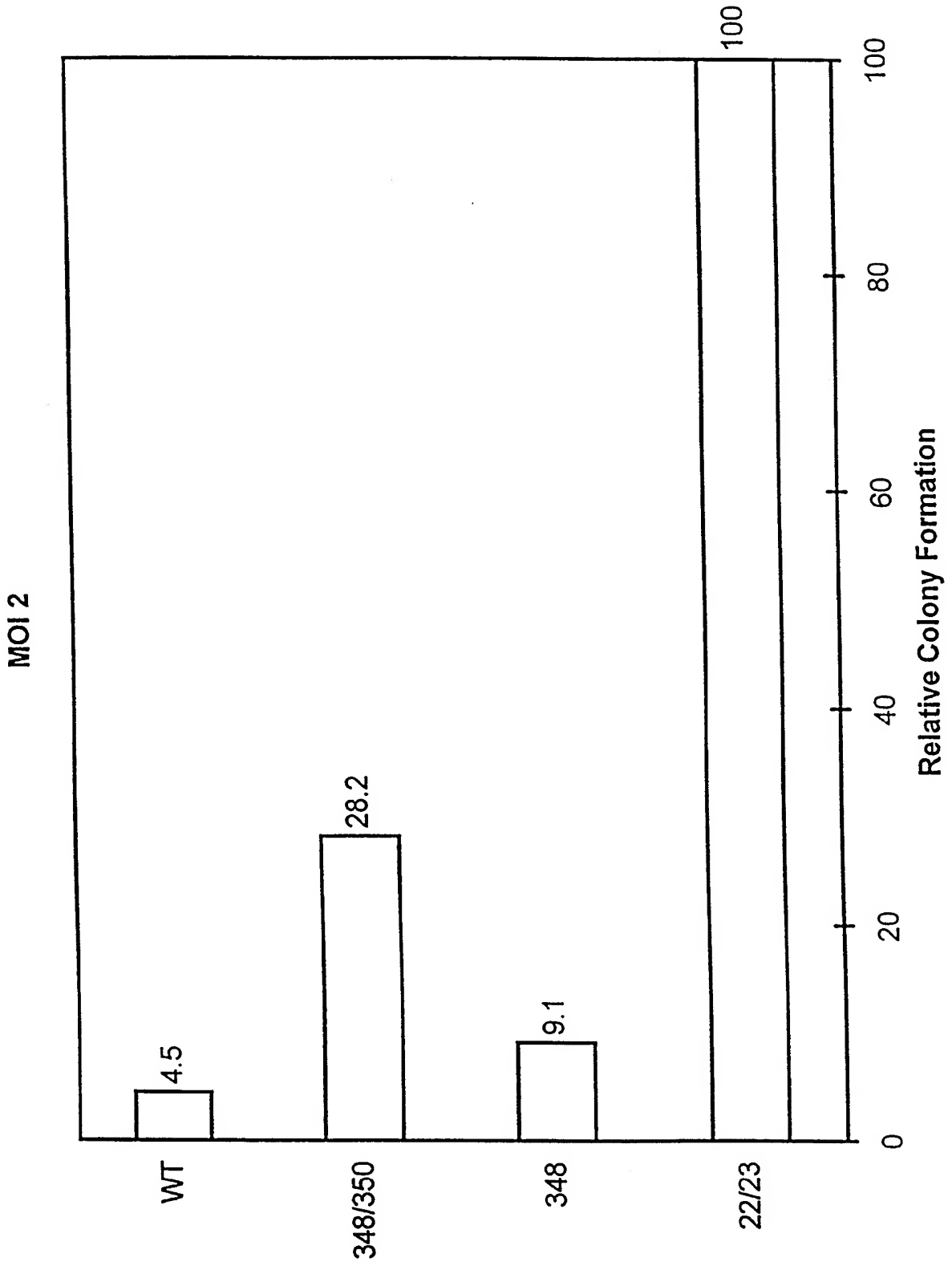
MOI 2
Relative Colony Formation
0
20
40
60
80
100
4.5
28.2
9.1
100
WT
348/350
348
22/23

Figure 6A

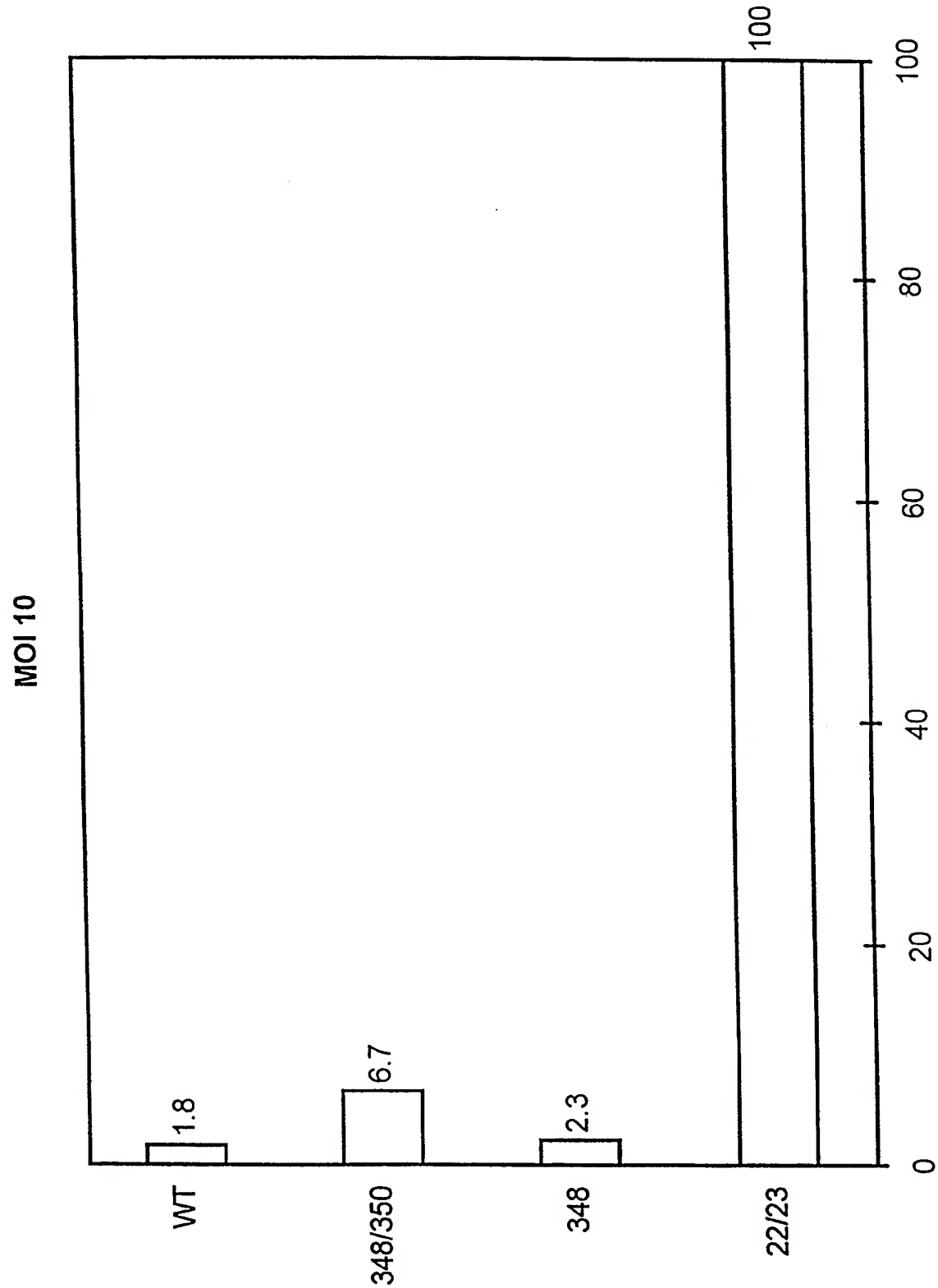
MOI 10
Relative Colony Formation
0
20
40
60
80
100
100
1.8
6.7
2.3
WT
348/350
348
22/23

Figure 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10252

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/00, 48/00; C12N 15/11, 15/63; C07H 21/04
US CL :424/93.2; 435/320.1; 514/44; 536/23.1, 23.4, 23.5,
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2; 435/320.1; 514/44; 536/23.1, 23.4, 23.5,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MIDDELER, G. et al. The tumor suppressor p53 is subject to both nuclear import and export, and both are fast, energy-dependent and lectin-inhibited. Oncogene. March 1997, Vol. 14, No. 12, pages 1407-1417, see entire document.	1-29
A,P	TAO, W.K. et al. Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. Proceeding of The National Acadmy of Sciences USA. March 1999, Vol. 96, No. 6, pages 3077-3080, see entire document.	1-29
Y	ROTH, J. et al. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. EMBO Journal. January 1998, Vol. 17, No. 2, pages 554-564, see entire document.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 JULY 1999	Date of mailing of the international search report 23 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUMESH KAUSHAL Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10252

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	STOMMEL, J.M. et al. A Leucine-rich Nuclear Export Signal in The p53 Tetramerization Domain: Regulation of Subcellular Localization and p53 Activity by NES Masking. EMBO journal. March 1999, Vol. 18, No. 6, pages 1660-1672, see entire document.	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10252

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN/CAS: SCISEARCH, MEDLINE, BIOSIS, CANCERLIT, BIOSIS. INPADOC
Search terms: Nuclear export, p53, P73, KET, RB1, WT1,nf1, VHL, APC, DCC, NB1, MLM, MEN1, MEN2A, BCNS, RCC, BRCA1, BRCA2, ETS1, CANCER, NEOPLAST, TUMOR, MALIGNANT, PEPTIDE